

Alteration of Reproductive Function via Activation of HMGB1/RAGE/TLR4 Signaling Pathway in the Testis of Dysbiosis-induced Mice

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Abstract

High-mobility group box 1 (HMGB1), released under conditions of cellular stress or damage, acts as a damage-associated molecular pattern (DAMP) that mediates inflammation through receptors such as the receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4). This study investigated the involvement of HMGB1, RAGE, and TLR4 signaling in testicular inflammation and reproductive impairment in dysbiosis-induced mice. Mice were provided either water containing antibiotics to induce dysbiosis (WD) or water without antibiotics (control; WO) for 4 weeks. Serum and reproductive organs were collected for analysis. Dysbiosis was associated with decreased serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels. In contrast, testicular levels of interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α were increased. Serum and testicular levels of HMGB1, HMGB2, and RAGE were also elevated. RT-PCR and Western blot analyses revealed upregulated expression of HMGB1, RAGE, TLR4, and cytokines, with reduced mRNA expression of spermatogenic and tight junction-related genes. Increased germ cell apoptosis and enhanced HMGB1-RAGE co-localization within the seminiferous epithelium were observed. Mating assays showed reduced pregnancy rates, litter sizes, and offspring birth weights. Collectively, these findings suggest that dysbiosis activates the HMGB1-RAGE-TLR4 axis, contributing to testicular inflammation, impaired spermatogenesis, and reduced fertility.

1. Introduction

The testis is a unique organ in which immune privilege coexists with metabolically demanding processes such as spermatogenesis and steroid hormone production [1]. This important balance is controlled by a tightly regulated network of immune and metabolic signaling pathways, a field now acknowledged as testicular immunometabolism [2]. Immune tolerance within the testicular microenvironment is preserved by the integrity of the blood-testis barrier (BTB), local immune cells, and immunoregulatory cytokines [3]. However, disturbances in immune-metabolic homeostasis can initiate inflammatory responses that compromise germ cell survival, disrupt hormonal signaling, and impair sperm cell development [4]. Testicular immune activation at the molecular level is commonly induced by the release of damage-associated molecular patterns (DAMPs) released from stressed or dying cells, which are subsequently recognized by innate pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE), leading to the initiation of local immune responses [5]. High-mobility group box 1 (HMGB1) is a well-characterized DAMP that binds chromatin in the nucleus and can be released extracellularly under conditions of cellular stress or injury [6, 7]. Upon release into the extracellular space, HMGB1 functions as a potent pro-inflammatory signal, binding to PRRs such as TLR4 and RAGE. These signals activate intracellular pathways, leading to the activation of nuclear factor-kappa B (NF- κ B), the production of pro-inflammatory cytokines, and the recruitment of immune cells [8]. The HMGB1-RAGE-TLR4 signaling axis is a central mediator of sterile inflammation and has been associated with reproductive pathologies, including testicular torsion [9], varicocele [10], and autoimmune orchitis [11]. Prolonged activation of this pathway disrupts the immune-privileged environment within the testis, disrupts the integrity of the BTB, and triggers germ cell apoptosis [12].

Furthermore, the interplay between immune activation and metabolic stress can aggravate inflammation, ultimately contributing to chronic testicular dysfunction. Gut dysbiosis, induced by factors such as antibiotic overuse, a high-fat diet, and exposure to environmental pollutants, including microplastics, can lead to impaired microbial metabolite production, increased gut permeability, and systemic translocation of endotoxins [13, 14]. Recent evidence suggests that signals derived from the gut microbiota, such as short-chain fatty acids, lipopolysaccharides, and other microbial metabolites, are key modulators of systemic and reproductive immunity [15, 16]. Alterations in these signals may induce a low-grade systemic inflammation that extends to the testis, activating PRRs such as TLR4 and RAGE, thereby linking gut dysbiosis to testicular immune activation. Thus, disruptions in microbial composition may lead to molecular alterations within the testis and impact fertility outcomes. A better understanding of the molecular pathways involved in immune-mediated reproductive dysfunction is essential to enhance therapeutic strategies in dysbiosis-induced infertility. Given these considerations, the present study investigated HMGB1, RAGE, and TLR4-mediated inflammatory responses and immunometabolic alterations in the testes of dysbiosis-induced mice to characterize the molecular mechanisms driving testicular dysfunction and microbiota-associated male subfertility and infertility.

2. Results

2.1 Effects of Dysbiosis on Serum Reproductive Hormone and Endotoxin Levels, and Hepatotoxicity

Antibiotic-induced dysbiosis significantly impaired reproductive hormone homeostasis (Fig. 1). Serum testosterone, LH, and FSH levels were significantly reduced in WD mice compared with controls (WO, $P < 0.05$; Fig. 1A-C). Furthermore, hepatotoxicity (serum GOT and GPT) and endotoxin levels were significantly increased in WD mice compared with controls (WO), confirming dysbiosis-associated systemic stress ($P < 0.05$, $P < 0.01$; Supplementary data 1A-C).

2.2 Effects of Dysbiosis on Pro-inflammatory Cytokine Levels in Serum and Testis

Dysbiosis induced a robust inflammatory response at both systemic and testicular levels. WD mice exhibited significantly elevated levels of IL-1 β , IL-6, and TNF- α in their testis extracts, compared to the controls (WO, $P < 0.05$, $P < 0.01$; Fig. 1D-F). Consistently, serum concentrations of IL-1 β , IL-6, and TNF- α were also significantly increased in WD mice compared to the controls (WO), indicating systemic inflammatory activation in both serum and testis caused by dysbiosis ($P < 0.05$, $P < 0.01$; Supplementary data 2A-C).

2.3 Effects of Dysbiosis on Testicular Levels of Inflammatory Mediators HMGB1, HMGB2, and RAGE

Testicular levels of HMGB1 and HMGB2 were significantly higher in WD mice than those of the controls (WO, $P < 0.05$; Fig. 1G&H). Furthermore, testicular level of RAGE, the principal receptor for HMGB proteins, was also significantly elevated compared to the controls (WO), indicating activation of HMGB-RAGE signaling within the testicular microenvironment ($P < 0.05$; Fig. 1I). Consistent with testicular alterations, serum levels of HMGB1, HMGB2, and RAGE were also significantly increased in the serum of WD mice compared to the controls (WO), further supporting dysbiosis-associated inflammatory activation ($P < 0.05$, $P < 0.01$; Supplementary data 2D-F).

2.4 Effects of Dysbiosis on Sperm Function and Inflammatory Marker Distribution

WD mice exhibited a significant reduction in sperm motility ($P < 0.05$; Fig. 2A) and sperm concentration ($P < 0.05$; Fig. 2B), compared to the controls (WO) without dysbiosis. Immunofluorescence analysis revealed that HMGB1, RAGE, and TLR4 were predominantly localized to the sperm tail region (Fig. 2C). Interestingly, spermatozoa derived from the WD group exhibited a somewhat stronger fluorescent intensity in the tail region (b-b" in Fig. 2C) compared to the control group (a-a" in Fig. 2C).

2.5 Effects of Dysbiosis on Molecular Signaling Pathways in Testicular Inflammation

To further investigate the molecular responses associated with dysbiosis-induced inflammation in the testis, the mRNA expression levels of pro-inflammatory cytokines were analyzed using Quantitative real-time PCR (qPCR, Fig. 3). As shown in Fig. 3A-C, the expressions of major pro-inflammatory cytokines *IL-1 β* , *IL-6*, and *TNF- α* were significantly upregulated in the testis of WD mice than those of the control group (WO; $P < 0.05$). Similarly, the mRNA expressions of *HMGB1*, *HMGB2*, and *RAGE* were significantly increased in the WD mice compared to the controls (WO), indicating the activation of the DAMP-receptor axis in response to the dysbiosis conditions ($P < 0.05$; Figs. 3D-F). Also, the expression levels of *TLR4* and *TLR2*, which are known to interact with HMGB proteins, were significantly higher in the WD group than the controls (WO, $P < 0.05$; Fig. 3G & H).

2.6 Effects of Dysbiosis on the Expression of Genes Associated with Steroidogenesis in the Testis

Additionally, the effect of dysbiosis-induced inflammation on testicular function, as well as the mRNA expression levels of genes involved in testicular development, BTB regulation, and steroidogenesis, were analyzed (Fig. 4). The expression levels of solute carrier family 38 member 5 (*Slc38a5*), desert hedgehog signaling molecule (*Dhh*), Musashi RNA Binding Protein 1 (*Msi1*), espin (*Espn*), GATA binding protein 4 (*Gata4*), steroidogenic acute regulatory protein (*STAR*), SRY-box transcription factor 9 (*SOX9*), and aquaporin 8 (*Aqp8*) were significantly downregulated in the WD group compared to the control group (WO, $P < 0.05$, $P < 0.01$; Fig. 4A-H).

2.7 Effects of Dysbiosis on BTB Integrity and Histology of Reproductive Tissues

To assess the impact of dysbiosis on BTB integrity, mRNA expression of tight junction proteins (TJPs) was analyzed by RT-PCR (Fig. 5A-F). Compared to the control group (WO), the WD group showed a significantly downregulated expression of the major BTB-associated tight junction proteins claudin-3, claudin-11, occludin, tight junction protein (TJP)1, TJP2, and cadherin 1, suggesting disruption of the BTB structure under conditions of gut microbiota imbalance ($P < 0.05$, $P < 0.01$; Fig. 5A-F). Histological analysis corroborated the molecular findings, revealing notable structural alterations in the reproductive tissues of WD mice (Fig. 5G). Testis sections from WD mice displayed disrupted seminiferous tubule architecture and reduced germinal epithelium height compared to the well-organized tubules observed in the WO group (a-a' in Fig. 5G). Similarly, the epididymis of the WD mice exhibited decreased luminal sperm content and epithelial disorganization (b-b' in Fig. 5G). Seminal vesicles in the WD group appeared atrophied, with thinning of the epithelial layer and reduced luminal secretion, indicating impaired accessory gland function (c-c' in Fig. 5G).

2.8 Effects of Dysbiosis Observed on Western Blot analysis of HMGB1, HMGB2, RAGE, and TLR4 in Testis

The western blot analysis of testis samples from the control (WO1-3) and dysbiosis-induced (WD1-3) groups using anti-HMGB1, anti-HMGB2, anti-RAGE, and anti-TLR4 antibodies revealed distinct bands at the expected molecular weights: 25 kDa for HMGB1, 24 kDa for HMGB2, 45 kDa for RAGE, and approximately 42 kDa for TLR4, respectively (Fig. 6A). β -tubulin (55 kDa) served as the reference protein for determining relative band density. Compared to the control group (WO), the band from the WD group exhibited significantly higher protein density of HMGB1 ($P < 0.001$; Fig. 6B), HMGB2 ($P < 0.01$; Fig. 6C), and RAGE ($P < 0.01$; Fig. 6D), consistent with the gene expression data. Moreover, TLR4 expression was markedly elevated in the WD group compared to the WO group ($P < 0.001$; Fig. 6E).

2.9 Effects of Dysbiosis on Testicular Apoptosis and HMGB1-RAGE Interaction

Compared to the control group (WO; a-a" in Fig. 7A), the testis sections from dysbiosis-induced mice (WD; b-b" in Fig. 7A) exhibited markedly increased red fluorescence signals, indicating a higher number of TUNEL-positive apoptotic cells (white arrows, b in Fig. 7A). The merged images revealed strong co-localization of apoptotic signals with DAPI-stained nuclei, particularly within the seminiferous tubules, suggesting that dysbiosis promotes germ cell apoptosis in the testis (a" & b" in Fig. 7A). To investigate the interaction between HMGB1 and its receptor RAGE within the testicular tissue, we performed an *in-situ* PLA on testis sections from the control (WO; a-a" in Fig. 7B) and dysbiosis-induced (WD; b-b" in Fig. 7B) mice. In the WD group, there was a marked increase in red fluorescence signal intensity, indicating enhanced co-localization and interaction between HMGB1 and RAGE (white arrows, b in

Fig. 7B). Remarkably, these interactions were predominantly localized within the seminiferous tubules, suggesting that the HMGB1-RAGE signaling axis is actively engaged at sites critical for spermatogenesis. In contrast, minimal PLA signals were detected in the WO group (a in Fig. 7B). The localization of this interaction in the germinal epithelium implies that DAMP-mediated signaling may directly influence the seminiferous microenvironment, potentially impairing Sertoli-germ cell communication and contributing to the apoptosis and disruption of spermatogenesis observed under dysbiosis conditions.

2.10 Effects of Dysbiosis on Reproductive Outcomes in Dysbiosis-Induced Mice

Mating assays showed that WD males had significantly reduced fertility. Females mated with WD males had a lower pregnancy rate (55.4% vs. 87.5%), smaller litter sizes (6.0 vs. 8.6 pups), and decreased pup birth weights (1.08 g vs. 1.58 g) compared to females mated with WO males ($P < 0.05$, $P < 0.01$; Supplementary data 3A-C).

3. Discussion

This study investigated the molecular interplay between gut microbiota dysbiosis and male reproductive dysfunction, with a focus on the HMGB1-RAGE-TLR4 signaling axis in testicular inflammation. Our findings demonstrate that antibiotic-induced microbial imbalance leads to systemic inflammation, endocrine disruption, and impaired testicular architecture and function.

Dysbiosis increased HMGB1 levels in both serum and testis, consistent with elevated inflammatory responses. HMGB1, a well-known alarmin and DAMP, is upregulated in response to cellular stress and triggers local and systemic inflammation by interacting with receptors such as RAGE and TLR4, promoting cytokine production [17, 18]. Elevated HMGB1 has been linked to impaired sperm function and testicular damage, consistent with our observations [19]. Serum IL-1 β , IL-6, and TNF- α were also increased, reflecting systemic inflammatory activation likely driven by gut barrier disruption and bacterial antigen translocation [20].

Hepatic stress, reflected by elevated GOT and GPT activities, indicates activation of the gut-liver-immune axis and its contribution to systemic inflammation [21]. The hypothalamic-pituitary-gonadal (HPG) axis regulates male gonadal function through hypothalamic gonadotropin-releasing hormone (GnRH)-mediated stimulation of pituitary FSH and LH secretion [22]. Pro-inflammatory cytokines are known to disrupt HPG axis signaling, leading to reduced FSH, LH, and testosterone levels, as well as impaired Leydig cell steroidogenesis [23, 24]. Consistent with these effects, we observed downregulation of *STAR* and *GATA4* in the testis, indicating impaired cholesterol transport and steroid hormone biosynthesis [25, 26].

Our data indicate that the testis responds directly to systemic immune stress through HMGB1-RAGE-TLR4 activation (Figs. 1 & 3). Upregulation of HMGB1, along with its receptors RAGE and TLR4 in testicular tissues, suggests a DAMP-driven inflammatory response within the seminiferous epithelium [27, 28]. RAGE expressed in Sertoli and germ cells amplifies inflammation by sustaining NF- κ B activation upon binding to HMGB1 [9]. Immunofluorescence and western blot analyses revealed increased testicular expression of RAGE and TLR4 (Figs. 2 & 6), indicating their involvement in innate immune sensing of dysbiosis-associated signals. This upregulation suggests that the testis actively participates in immune activation in response to systemic endotoxin exposure [29]. TLR4 signaling is known to disrupt tight junctions and impair BTB integrity via MyD88-dependent pro-inflammatory pathways [30, 31]. Moreover, elevated TLR2 expression suggests broader engagement of pattern recognition receptors in the testis during dysbiosis, consistent with microbiota-driven immune activation reported in other tissues [32].

Molecular and histological analyses revealed significant downregulation of tight junction proteins (claudin-3, claudin-11, occludin, TJP1, TJP2, cadherin-1), reflecting impaired BTB integrity and facilitating the infiltration of immune cells and harmful molecules to the seminiferous epithelium [33].

Disruption of Sertoli cell-germ cell interactions, accompanied by reduced *Msi1* and *Espn* expression, indicates Sertoli cell dysfunction, which is essential for maintenance of spermatogenesis [34, 35]. The intrinsic mitochondrial apoptotic pathway was activated, as evidenced by upregulated *Bax* and *caspase-3* and decreased *Bcl-2* expression [36]. *In situ* PLA confirmed direct HMGB1-RAGE interactions within the seminiferous tubules, highlighting a mechanism whereby DAMP signaling triggers germ cell apoptosis and disrupts spermatogenesis [11].

Sperm function was also impaired, with reduced motility and concentration, and elevated levels of HMGB1, RAGE, and TLR4 in epididymal spermatozoa (Fig. 2). These results suggest that inflammatory mediators not only disrupt spermatogenesis but also persist in the epididymis, thereby affecting sperm maturation [37]. Histological examination of accessory glands revealed thinning of the epithelium and reduced secretory activity in the seminal vesicles, indicating that dysbiosis-related inflammation extends beyond the testis, further impairing male fertility.

Consistent with the molecular and histological findings, WD males exhibited significantly reduced pregnancy rates and litter sizes, and pups sired by WD males had lower birth weights (Supplementary data 3), demonstrating that paternal dysbiosis can adversely affect reproductive success and offspring development [38].

Taken together, our study demonstrates that the activation of the HMGB1-RAGE-TLR4 signaling axis emerges as a key mechanism linking intestinal microbial dysbiosis to testicular inflammation, BTB disruption, and germ cell apoptosis. Dysbiosis-induced upregulation of HMGB1 and its receptors, along with elevated pro-inflammatory cytokines, highlights the role of DAMP-mediated immune activation in testicular dysfunction. The simultaneous downregulation of tight junction and steroidogenic genes, combined with seminiferous epithelium disruption and reduced sperm motility, confirms the adverse

impact of gut microbial imbalance on male fertility. Furthermore, reduced pregnancy rates, smaller litter sizes, and lower pup birth weights in dysbiosis-induced males provide functional validation of the observed molecular and cellular disturbances. Collectively, these findings establish a mechanistic link between gut dysbiosis and reproductive impairment, emphasizing the gut-testis axis as a critical mediator in male reproductive health.

4. Materials and Methods

4.1 Animals and Induction of Dysbiosis

Forty 9-week-old male C57BL/6 mice (Samtaco, Korea) were acclimated for 1 week under standard conditions with ad libitum access to water and diet. Mice were assigned to two groups (n = 20 each); one received antibiotics dissolved in deionized/distilled water (1 g/L ampicillin, 1 g/L neomycin sulfate, 1 g/L vancomycin, 500 mg/L metronidazole) to induce dysbiosis (WD) [39], and the other received water without antibiotics (WO) for 4 weeks. Body weight and water intake were monitored weekly. All animal procedures were performed in accordance with the Animal Care and Use Committee (ACUC) guidelines and were approved by the ACUC of Sunchon National University (SCNU IACUC-2025-02). Unless otherwise specified, all reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2 Mating and Determination of Fertility Competence

After the 4-week experimental period, 6 males per group were paired with fertile female C57BL/6 mice at a 1:2 male-to-female ratio for 3–5 days. Females were examined daily for the presence of copulation plugs and subsequently housed individually until delivery. Fertility outcomes, including the pregnancy rate and litter size, were used to evaluate the reproductive competence of the WD and WO male mice.

4.3 Sample Preparation for Analysis

Animals were euthanized by CO₂ inhalation after the 4-week experimental period, and blood and reproductive organs were immediately collected. Serum was separated by centrifugation at 1500 × g for 10 min and stored at -20°C. Organs were preserved in RNAiso (Takara Bio Inc., Shiga, Japan) at -80°C before RNA extraction.

4.4 Hepatotoxicity and Endotoxin Assay

Levels of serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured using the manufacturer's instructions for the reagent kits (GOT/GPT kits, Asan Pharm. Co., Ltd., Seoul, Korea). Serum endotoxin levels were measured using the Pierce™ Chromogenic LAL Endotoxin Quantitation Kit (#88282, Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

4.5 Assessment of Reproductive Hormone Levels

Serum levels of testosterone were measured using the competitive enzyme immunoassay kit (#582701, Cayman Chemical, Ann Arbor, MI, USA), while luteinizing hormone (LH) (#MBS8427398, MyBioSource, San Diego, CA, USA) and follicle-stimulating hormone (FSH) (#MBS2700327, MyBioSource) levels were determined using the sandwich enzyme linked immunosorbent assay (ELISA) principle according to the manufacturer's instructions.

4.6 Assessment of Inflammatory Cytokines and Mediators

Total protein concentrations in serum and testis homogenates were measured by the Bradford assay, with bovine serum albumin (BSA) as the standard. Interleukin (IL)-1 β was measured using the ELISA MAX[™] Standard Set (BioLegend, San Diego, CA, USA), while IL-6 and tumor necrosis factor (TNF)- α were quantified using ELISA kits from Thermo Fisher Scientific (USA). HMGB1 (Chondrex, Inc., Woodinville, WA, USA), HMGB2 (#MBS2885784, MyBioSource), and RAGE were quantified using the Quantikine[®] Mouse RAGE ELISA kit (R&D Systems, Minneapolis, MN, USA). All assays were performed both in serum and testicular homogenates according to the manufacturer's protocols.

4.7 Analysis of Sperm Quality and Immunofluorescence of Epididymal Spermatozoa

Epididymal spermatozoa were harvested by dissecting the caput, corpus, and cauda, followed by gentle pipetting in phosphate-buffered saline (PBS). Suspensions were filtered through 40 μ m strainers (SPL Life Sciences Co., Ltd., Korea) and centrifuged at 300 $\times$ g for 15 min at 4°C. Sperm motility parameters were measured with a computer-assisted sperm analyzer (CASA, Sperm Class Analyzer, Microptic S.L., Barcelona, Spain), and sperm concentration was determined using a hemocytometer. For immunofluorescence staining, spermatozoa were mounted on poly-L-lysine-coated coverslips in KMT medium (100 mM KCl, 2 mM MgCl₂, 10 mM TRIS-HCl, pH 7.0), fixed with 2% formaldehyde, permeabilized with PBS containing 0.1% Triton-X 100 (PBS-TX), and blocked with 5% normal goat serum (NGS). Spermatozoa were then incubated with primary antibodies, followed by fluorophore-conjugated secondary antibodies. DNA was labeled with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Thermo Fisher Scientific), and all immunofluorescence images were captured using a fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA). Detailed antibody information is provided in Supplementary Table 1.

4.8 Real-time PCR Analysis

Total RNA was extracted from testis samples using RNAiso Plus (Takara) according to the manufacturer's instructions. The RNA concentration was determined using a Nanodrop spectrophotometer (DeNovix DS-11FX, Wilmington, NC, USA). The cDNA was synthesized using the ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Real-time PCR assays were performed in triplicate using SYBR[™] Premix Ex Taq[™] II (Bioneer, Daejeon, Korea) on a MyGo Pro PCR system (Diagnostic Technology, Belrose, Australia). The expression levels of the target genes were normalized to the housekeeping gene β -actin. Primers for inflammatory, tight junction,

and reproductive genes were designed using the Primer-Basic Local Alignment Search Tool (BLAST) or obtained from previous studies [40–42]. All primer sequences are listed in Table 1.

Table 1
Primer sequences for RT-PCR

Gene name*	Primer sequence (5'-3')	
<i>IL-1β</i>	F: GCTGCTTCCAAACCTTTGAC	R: TTCTCCACAGCCACAATGAG
<i>IL-6</i>	F: CCGGAGAGGAGACTTCACAG	R: CAGAATTGCCATTGCACAAC
<i>TNF-α</i>	F: TGTCTCAGCCTCTTCTCATTCCTG	R: AGGCCATTTGGGAAGTTCTCATCC
<i>HMGB1</i>	F: CATCTCGGGGCCAAAGCGA	R: TGAGGACATTTTGCCTCTCGG
<i>HMGB2</i>	F: AAGAGGCTGGGGATTGCGTT	R: TACCTGCCGAGGAGAAAGCC
<i>RAGE</i>	F: ACATACAAGATGGACCTGTGGA	R: CCAGTTCATTACACCAGGA
<i>TLR4</i>	F: ACTCAGCAAAGTCCCTGATGACA	R: AGGTGGTGTAAAGCCATGCCA
<i>TLR2</i>	F: GGTGCCCAGATGGCTAGTGG	R: CAGAAGGAAACAGTCCGCACC
<i>Sc138a5</i>	F: GCTGCTCTTTCCAAGCAAGG	R: TTGGCACACAGATGACGAGG
<i>Dhh</i>	F: TTGGCTTGGGTGTATCTGTGG	R: GAACGGTAGATGGGGAACCA
<i>Msi 1</i>	F: GAGGACTCAGTTGGCAGACC	R: CCGCATCACCAGACACTCTT
<i>Espn</i>	F: CCTCCCAGCCTGAGTCACC	R: ATTGAGCAGTGGCTGAGACC
<i>Gata4</i>	F: GACACCCCAATCTCGATATGTT	R: CTTAATGAGGGGCCGGTTGA
<i>STAR</i>	F: GCGCTGGAGAGACCATTGTA	R: CCCAGCAGGAATCTCGTCTT
<i>Sox 9</i>	F: CACCAGTATCAGCGAGGAGG	R: GTCCAAACAGGCAGGGAGAT
<i>Aqp8</i>	F: AAGTGAGTACAATCTAGGGCAGC	R: CTACACATTGGTGTCTGCTCC
<i>Claudin-3</i>	F: GCACCCACCAAGATCCTCTA	R: TCGTCTGTCACCATCTGGAA
<i>Claudin-11</i>	F: TGGTGGACATCCTCATCCTT	R: GCCAGCAGAATAAGGAGCAC
<i>Occludin</i>	F: CCTACTCCTCCAATGGCAAA	R: CTCTTGCCCTTTCCTGCTTT
<i>TJP 1 (ZO-1)</i>	F: GCACCATGCCTAAAGCTGTC	R: ACTCAACACACCACCATTGC
<i>TJP 2 (ZO-2)</i>	F: AATGCGAGGATCGAAATAGC	R: TAGCTTCCTCTGGTGTCTCTG
<i>Cadherin-1</i>	F: ACGTCCATGTGTGTGACTGTG	R: AGGAGCAGCAGGATCAGAAC
<i>Actin, beta</i>	F: TGTTACCAACTGGGACGACA	R: GGGGTGTTGAAGGTCTCAAA

***Abbreviations:** IL, Interleukin; TNF- α , Tumor necrosis factor; HMGB, High-mobility-group-box; RAGE, Receptor for advanced glycation end products; TLR, Toll-like receptor; Sc138a5, Solute carrier family 38 member 5; Dhh, Desert hedgehog signaling molecule; Msi 1, Musashi RNA Binding Protein 1; Espn, espin;

Gata4, GATA-binding factor 4; STAR, Steroidogenic acute regulatory protein; Sox 9, SRY-box transcription factor 9; Aqp8, Aquaporin 8; TJP1&2, tight junction protein 1&2.

4.9 Histopathological Evaluation of Reproductive Tissues

Tissue samples of the testis, epididymis, and seminal vesicle were fixed in 4% buffered formaldehyde (Sigma-Aldrich). The tissues were subsequently dehydrated through a graded ethanol series, cleared with xylene, and embedded in paraffin. Then sections (5 μ m) were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). The stained tissue sections were mounted with a mounting medium and examined under a light microscope (Nikon).

4.10 Western Blot Assay

Proteins were extracted from the testis samples with an ice-cold lysis buffer, and protein concentrations were quantified using the Bradford assay. Equal amounts of proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk prepared in tris-buffered saline (TBS) containing Tween-20 and incubated overnight at 4°C with primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr. at room temperature (RT). The immunoreactive bands were visualized using the SuperSignalTM West Femto Maximum Sensitivity Substrate (#34096, Thermo Fisher Scientific) and captured using Davinch-Chemi Fluoro imaging system (Davinch-K Co., Ltd, Seoul, Korea). Details of the primary and secondary antibodies used are provided in Supplementary Table 1.

4.11 Terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) Assay

Apoptotic cells in testis tissues were assessed using the In Situ Cell Death Detection Kit, TMR red (Roche, Sigma-Aldrich), according to the manufacturer's instructions with slight modifications. Paraffin-embedded tissue sections were deparaffinized, rehydrated, and permeabilized with proteinase K (20 μ g/mL in PBS) at RT. Sections were then incubated with the Terminal deoxynucleotidyl transferase (TdT) UTP Nick-End Labeling (TUNEL) reaction mixture in a humidified chamber at 37°C for 1 hr. in the dark. Negative controls were processed without terminal deoxynucleotidyl transferase (TdT), while positive controls were treated with DNase I. Then, the slides were rinsed and counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. The sections were mounted using a mounting medium and analyzed under a fluorescence microscope equipped with the appropriate filter set for TMR (ex. 550 - em. 580 nm; Nikon).

4.12 In situ Proximity Ligation Assay

The co-localization of HMGB1 and RAGE in paraffin-embedded testis sections was examined using the in-situ proximity ligation assay (PLA) with the NaveniFlex Tissue GR Atto 647N kit (Navinci Diagnostics, Uppsala, Sweden), according to the manufacturer's protocol. Tissue sections were deparaffinized,

rehydrated, blocked, and incubated overnight at 4°C with primary antibodies against HMGB1 and RAGE. After incubation, the tissue sections were washed thoroughly and then incubated with the goat and rabbit Navenibodies mix (secondary antibodies conjugated to proprietary oligonucleotide probes), followed by ligation and rolling-circle amplification. To visualize protein-protein interactions, the sections were incubated with the rolling-circle amplification detection mix, and the nuclei were counterstained with DAPI. A fluorescence microscope (Nikon) was used to analyze PLA signals, indicating the proximity (co-localization) of HMGB1 and RAGE within the testicular tissue.

4.13 Statistical Analysis

Data were analyzed with GraphPad PRISM® (GraphPad Software, San Diego, CA, USA) and expressed as mean $\pm$ standard error of the mean (SEM). Comparisons among groups were performed using the unpaired two-tailed t-test. Each experiment was repeated three times independently. Differences were considered statistically significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Declarations

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Data Availability

All datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contribution

Wijesooriya Mudhiyanselage Nadeema Dissanayake: experiment, results, writing
Malavi Arachchi Gamage Nayodya Dananjane Malavi Arachchi Gamage: experiment, results
Sang-Myeong Lee: concept, experimental design, review
Young-Joo Yi: concept, experimental design, writing, review

References

1. Ye, L. et al. Impacts of immunometabolism on male reproduction. *Front. Immunol.* **12**, 658432. 10.3389/fimmu.2021.658432 (2021).
2. Carrageta, D. F. et al. Signatures of metabolic diseases on spermatogenesis and testicular metabolism. *Nat. Rev. Urol.* **21**, 477–494;10.1038/s41585-024-00866-y (2024).
3. Qu, N. et al. Immunological microenvironment in the testis. *Reprod. Med. Biol.* **19**, 24–31;10.1002/rmb2.12293 (2020).

4. Gong, J., Zeng, Q., Yu, D. & Duan, Y. G. T lymphocytes and testicular immunity: a new insight into immune regulation in testes. *Int. J. Mol. Sci.* **22**, 57;10.3390/ijms22010057 (2020).
5. Land, W. G. The role of damage-associated molecular patterns in human diseases: part I-promoting inflammation and immunity. *Sultan Qaboos Univ. Med. J.* **15**, e9 (2015).
6. Venereau, E. et al. HMGB1 as biomarker and drug target. *Pharmacol. Res.* **111**, 534–544. 10.1016/j.phrs.2016.06.031 (2016).
7. Deng, M. et al. The endotoxin delivery protein HMGB1 mediates caspase-11-dependent lethality in sepsis.. *Immunity.* **49**, 740–753. 10.1016/j.immuni.2018.08.016 (2018).
8. Li, P. & Chang, M. Roles of PRR-mediated signaling pathways in the regulation of oxidative stress and inflammatory diseases. *Int. J. Mol. Sci.* **22**, 7688. 10.3390/ijms22147688 (2021).
9. Aslani, F. High mobility group box protein-1 is involved in modulation of cell specific immune responses during chronic testicular inflammation in rat. PhD thesis, Justus-Liebig-Universität Giessen (2013).
10. Sereshki, N. et al. Decreased toll-like receptor (TLR) 2 and 4 expression in spermatozoa in couples with unexplained recurrent spontaneous abortion (URSA). *Iran. J. Allergy Asthma Immunol.* **18**, 701–706. 10.18502/ijaai.v18i6.2183 (2019).
11. Aslani, F. et al. Targeting high mobility group box protein 1 ameliorates testicular inflammation in experimental autoimmune orchitis. *Hum. Reprod.* **30**, 417–431. 10.1093/humrep/deu320 (2015).
12. Kaur, G., Wright, K., Verma, S. & Haynes, A. The good, the bad and the ugly of testicular immune regulation: a delicate balance between immune function and immune privilege. *Adv. Exp. Med. Biol.* 21–47. 10.1007/978-3-030-77779-1-2 (2021).
13. Ding, N. et al. Impairment of spermatogenesis and sperm motility by the high-fat diet-induced dysbiosis of gut microbes. *Gut* **69**, 1608–1619. 10.1136/gutjnl-2019-319127 (2020).
14. Guo, Q. et al. The gut microbiota contributes to the development of LPS-induced orchitis by disrupting the blood-testosterone barrier in mice. *Reprod. Sci.* **31**, 3379–3390. 10.1007/s43032-024-01613-9 (2024).
15. Li, B., Xiong, Y., Guo, D., Deng, G. & Wu, H. The gut-reproductive axis: bridging microbiota balances to reproductive health and fetal development. *Int. Immunopharmacol.* **144**, 113627. 10.1016/j.intimp.2024.113627 (2025).
16. Wang, N. et al. The effects of microbiota on reproductive health: a review. *Crit. Rev. Food Sci. Nutr.* **64**, 1486–1507. 10.1080/10408398.2022.2117784 (2024).
17. Andersson, U. & Tracey, K. J. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu. Rev. Immunol.* **29**, 139–162. 10.1146/annurev-immunol-030409-101323 (2011).
18. Dong, H., Zhang, Y., Huang, Y. & Deng, H. Pathophysiology of RAGE in inflammatory diseases. *Front. Immunol.* **13**, 931473. 10.3389/fimmu.2022.931473 (2022).
19. Elmorsy, E. H., Aly, R. G., Badae, N. M., Aboghazala, M. M. & Omar, S. S. Zinc alleviates high-fat diet-induced spermatogenic dysfunction in Wistar rats: role of oxidative stress, HMGB1 and

- inflammasome. *Rev. Int. Androl.* **22**, 44–52. 10.22514/j.androl.2024.007 (2024).
20. Di Vincenzo, F., Del Gaudio, A., Petito, V., Lopetuso, L. R. & Scaldaferri, F. Gut microbiota, intestinal permeability, and systemic inflammation: a narrative review. *Intern. Emerg. Med.* **19**, 275–293. 10.1007/s11739-023-03374-w (2024).
21. Chiou, W. C. et al. Gut microbiota-directed intervention with high-amylose maize ameliorates metabolic dysfunction in diet-induced obese mice. *Food Funct.* **13**, 9481–9495. 10.1039/D2FO01211A (2022).
22. Fischer, S., Ehlert, U. & Castro, R. A. Hormones of the hypothalamic-pituitary-gonadal (HPG) axis in male depressive disorders: a systematic review and meta-analysis. *Front. Neuroendocrinol.* **55**, 100792. 10.1016/j.yfrne.2019.100792 (2019).
23. Barabás, K., Szabó-Meleg, E. & Ábrahám, I. M. Effect of inflammation on female gonadotropin-releasing hormone (GnRH) neurons: mechanisms and consequences. *Int. J. Mol. Sci.* **21**, 529. 10.3390/ijms21020529 (2020).
24. Oduwole, O. O., Huhtaniemi, I. T. & Misrahi, M. The roles of luteinizing hormone, follicle-stimulating hormone and testosterone in spermatogenesis and folliculogenesis revisited. *Int. J. Mol. Sci.* **22**, 12735. 10.3390/ijms222312735 (2021).
25. Jahromi, M. S. et al. Elevated expression of steroidogenesis pathway genes CYP17, GATA6 and StAR in prenatally androgenized rats. *Gene* **593**, 167–171. 10.1016/j.gene.2016.07.067 (2016).
26. Manna, P. R. & Stocco, D. M. Regulation of the steroidogenic acute regulatory protein expression: functional and physiological consequences. *Curr. Drug Targets Immune Endocr. Metabol Disord.* **5**, 93–108. 10.2174/1568008053174714 (2005).
27. Yang, H., Wang, H., Chavan, S. S. & Andersson, U. High mobility group box protein 1: the prototypical endogenous danger molecule. *Mol. Med.* **21**, S6–S12. 10.2119/molmed.2015.00087 (2015).
28. Land, W. G. *Damage-associated molecular patterns in human diseases: volume 1: injury-induced innate immune responses* (Springer, 2018).
29. Bianchi, M. E. & Manfredi, A. A. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol. Rev.* **220**, 35–46. 10.1111/j.1600-065x.2007.00574.x (2007).
30. Wang, F., Chen, R. & Han, D. Male reproductive system and male fertility. In: *Innate immunity in health and disease*, p. 229 (2021).
31. Feng, R. et al. The ameliorative effect of melatonin on LPS-induced Sertoli cells inflammatory and tight junctions damage via suppression of the TLR4/MyD88/NF-κB signaling pathway in newborn calf. *Theriogenology* **179**, 103–116. 10.1016/j.theriogenology.2021.11.020 (2022).
32. Glavan, T. W. et al. Gut immune dysfunction through impaired innate pattern recognition receptor expression and gut microbiota dysbiosis in chronic SIV infection. *Mucosal Immunol.* **9**, 677–688. 10.1038/mi.2015.92 (2016).
33. Mruk, D. D. & Cheng, C. Y. The mammalian blood-testis barrier: its biology and regulation. *Endocr. Rev.* **36**, 564–591 (2015). 10.1210/er.2014 – 1101.

34. ErLin, S. et al. Musashi-1 maintains blood-testis barrier structure during spermatogenesis and regulates stress granule formation upon heat stress. *Mol. Biol. Cell* **26**, 1947–; (1956). 10.1091/mbc.E14-11-1497 (2015).
35. O'Donnell, L. et al. Sertoli cell-enriched proteins in mouse and human testicular interstitial fluid. *PLoS One*. **18**, e0290846. 10.1371/journal.pone.0290846 (2023).
36. Giannattasio, A. et al. RNA expression of Bcl-w, a new related protein Bcl-2 family, and caspase-3 in isolated Sertoli cells from pre-pubertal rat testes. *J. Endocrinol. Invest.* **25** (25), RC23–RC. 10.1007/BF03345074 (2002).
37. Lv, S. et al. Gut microbiota is involved in male reproductive function: a review. *Front. Microbiol.* **15**, 1371667. 10.3389/fmicb.2024.1371667 (2024).
38. Argaw-Denboba, A. et al. Paternal microbiome perturbations impact offspring fitness. *Nature* **629**, 652–659. 10.1038/s41586-024-07336-w (2024).
39. Ge, X. et al. Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. *J. Transl Med.* **15** 10.1186/s12967-016-1105-4 (2017).
40. Kuroda, N. et al. Apoptotic response through a high mobility group box 1 protein-dependent mechanism in LPS/GalN-induced mouse liver failure and glycyrrhizin-mediated inhibition. *PLoS One*. **9**, e92884. 10.1371/journal.pone.0092884 (2014).
41. Schrade, A. et al. GATA4 regulates blood-testis barrier function and lactate metabolism in mouse Sertoli cells. *Endocrinology* **157**, 2416–2431 (2016). 10.1210/en.2015 – 1927.
42. Chihara, M., Otsuka, S., Ichii, O., Hashimoto, Y. & Kon, Y. Molecular dynamics of the blood-testis barrier components during murine spermatogenesis. *Mol. Reprod. Dev.* **77**, 630–639. 10.1002/mrd.21200 (2010).

Figures

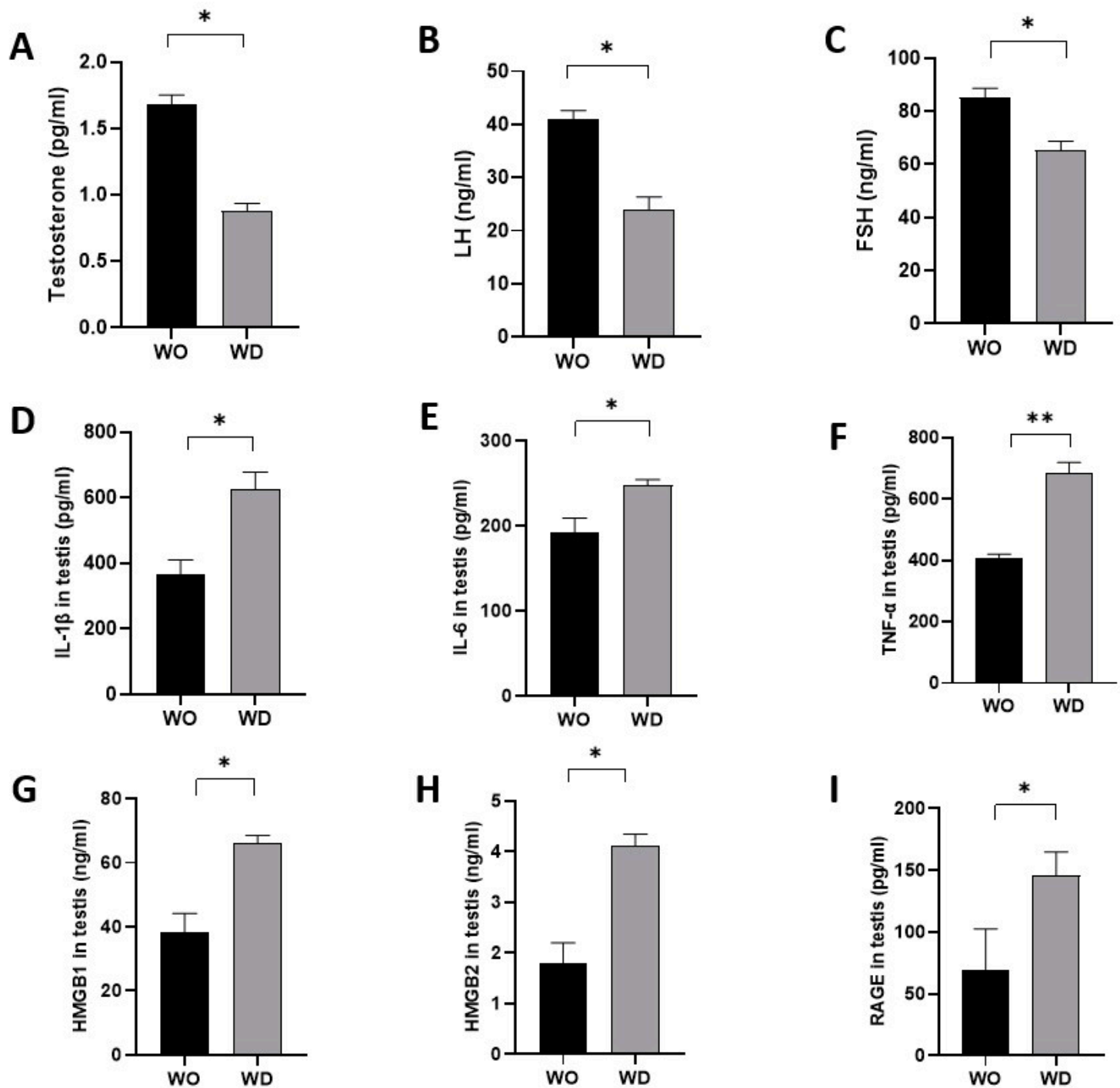


Figure 1

Testosterone (A), luteinizing hormone (LH) (B), and follicle-stimulating hormone (FSH) (C) levels in serum samples of the WO (control group) and WD (dysbiosis-induced group). The level of cytokines interleukin (IL)-1 β (D), IL-6 (E), and tumor necrosis factor (TNF)- α (F), high-mobility group box (HMGB)1 (G), HMGB2 (H), and receptor for advanced glycation end products (RAGE) (I) in the testicular extracts of the control (WO) and dysbiosis-induced groups (WD), respectively. Values are expressed as the mean $\pm$ SEM. The superscripts denote significance at *P<0.05 and **P<0.01.

Figure 2

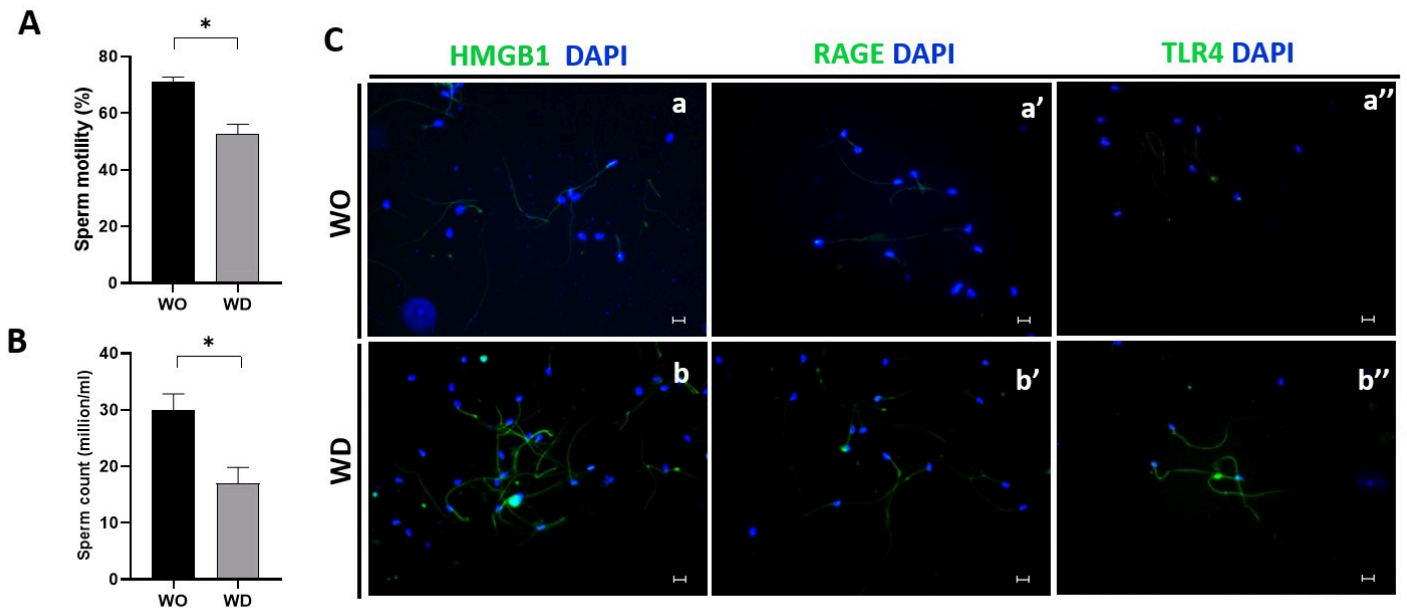


Figure 2

The motility (A) and number of spermatozoa (B) were examined in samples taken from the epididymis of both the control (WO) and dysbiosis groups (WD). Immunofluorescence staining revealed the localization of high-mobility group box 1 (HMGB1), receptor for advanced glycation end products (RAGE), and toll-like receptor 4 (TLR4) in the tail of epididymal spermatozoa (C). The fluorescence intensity was found to be more pronounced in the WD group (b-b'') compared to WO (a-a''). Values are expressed as the mean $\pm$ SEM. The superscripts denote significance at * $P < 0.05$ and ** $P < 0.01$. DAPI: 4',6-diamidino-2-phenylindole.

Figure 3

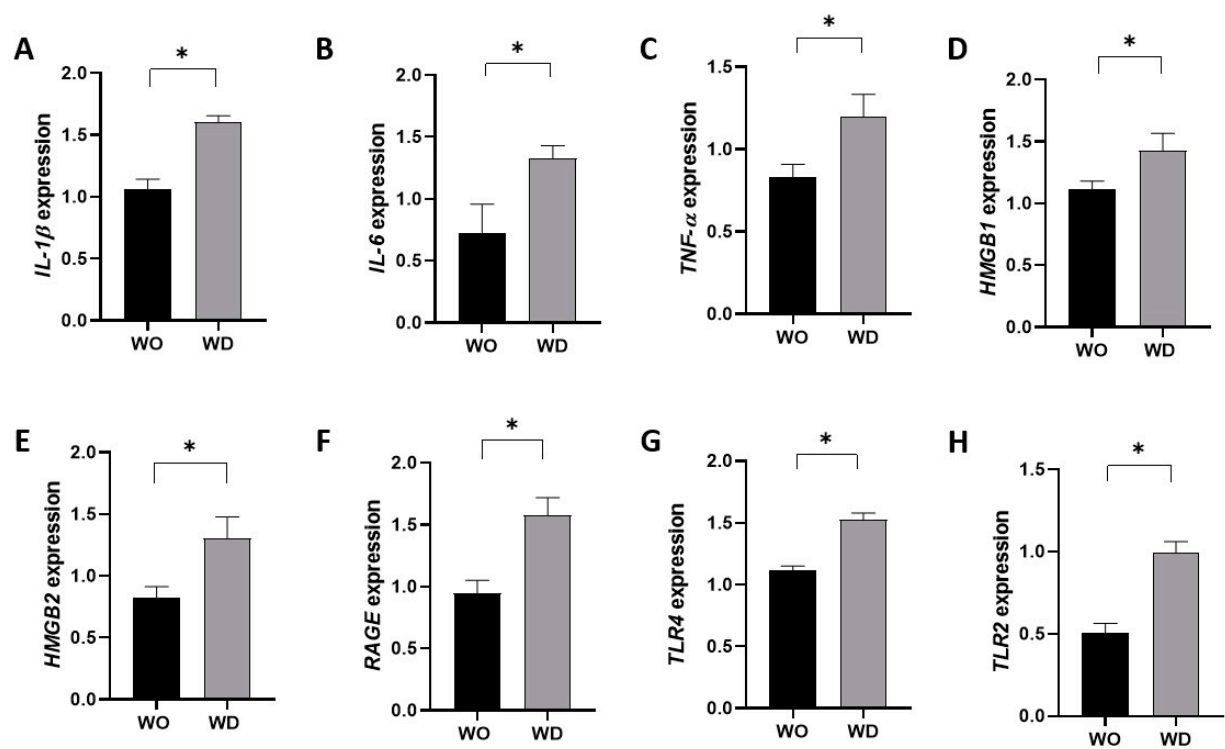


Figure 3

The relative mRNA expression of the following inflammatory cytokines and markers in the testicular extracts derived from the control group (WO) and dysbiosis group (WD); interleukin (*IL*)-1 β (A), *IL*-6 (B), tumor necrosis factor (*TNF*)- α (C), high-mobility group box 1 (*HMGB* 1) (D), *HMGB*2 (E), receptor for advanced glycation end products (*RAGE*) (F), toll-like receptor 4 (*TLR*4), (G) and *TLR*2 (H). Values are expressed as the mean $\pm$ SEM. The superscripts denote significance at *P<0.05 and **P<0.01.

Figure 4

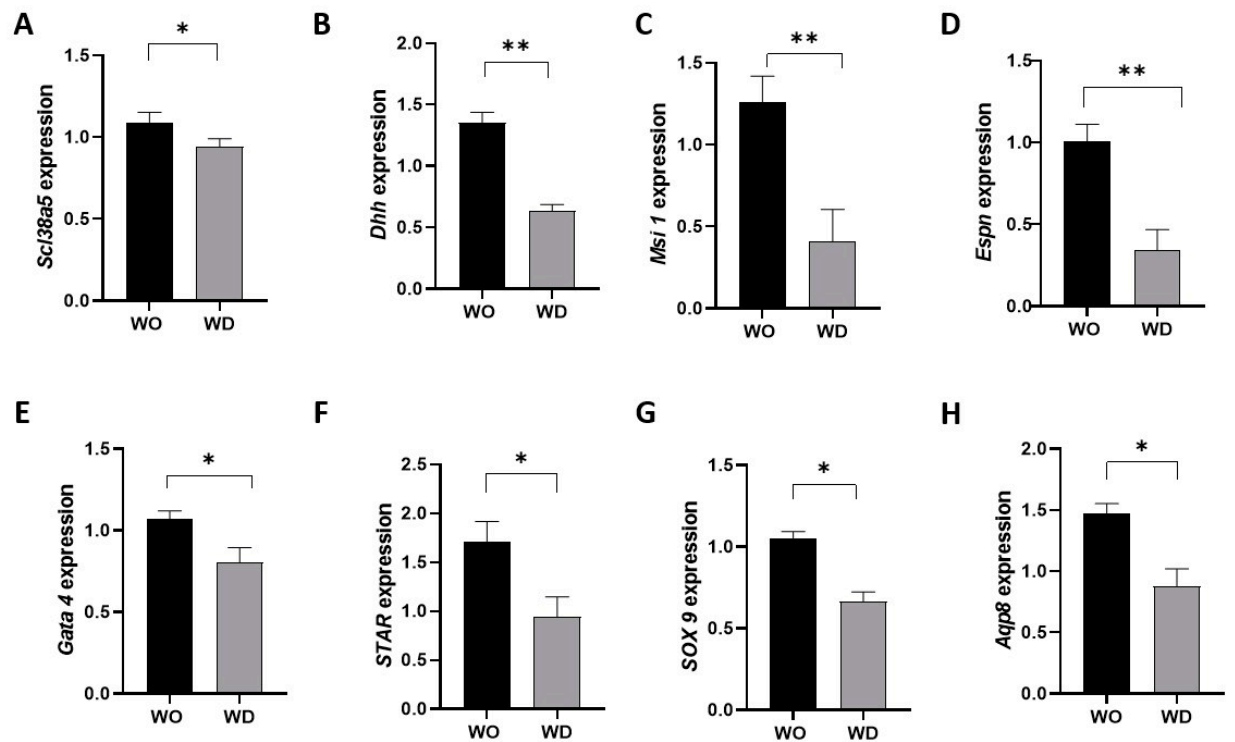


Figure 4

The relative mRNA expression levels associated with the Sertoli cell function and steroidogenesis in the testes of WO (control) and WD (dysbiosis-induced) mice; *Slc38a5* (solute carrier family 38 member 5; A), *Dhh* (Desert hedgehog signaling molecule; B), *Msi1* (Musashi RNA-binding protein 1; C), *Espn* (Espin; D), *Gata4* (GATA-binding protein 4; E), *STAR* (Steroidogenic acute regulatory protein; F), *SOX9* (SRY-box transcription factor 9; G), and *Aqp8* (Aquaporin 8; H). Values are expressed as the mean $\pm$ SEM. The superscripts denote significance at * $P < 0.05$ and ** $P < 0.01$.

Figure 5

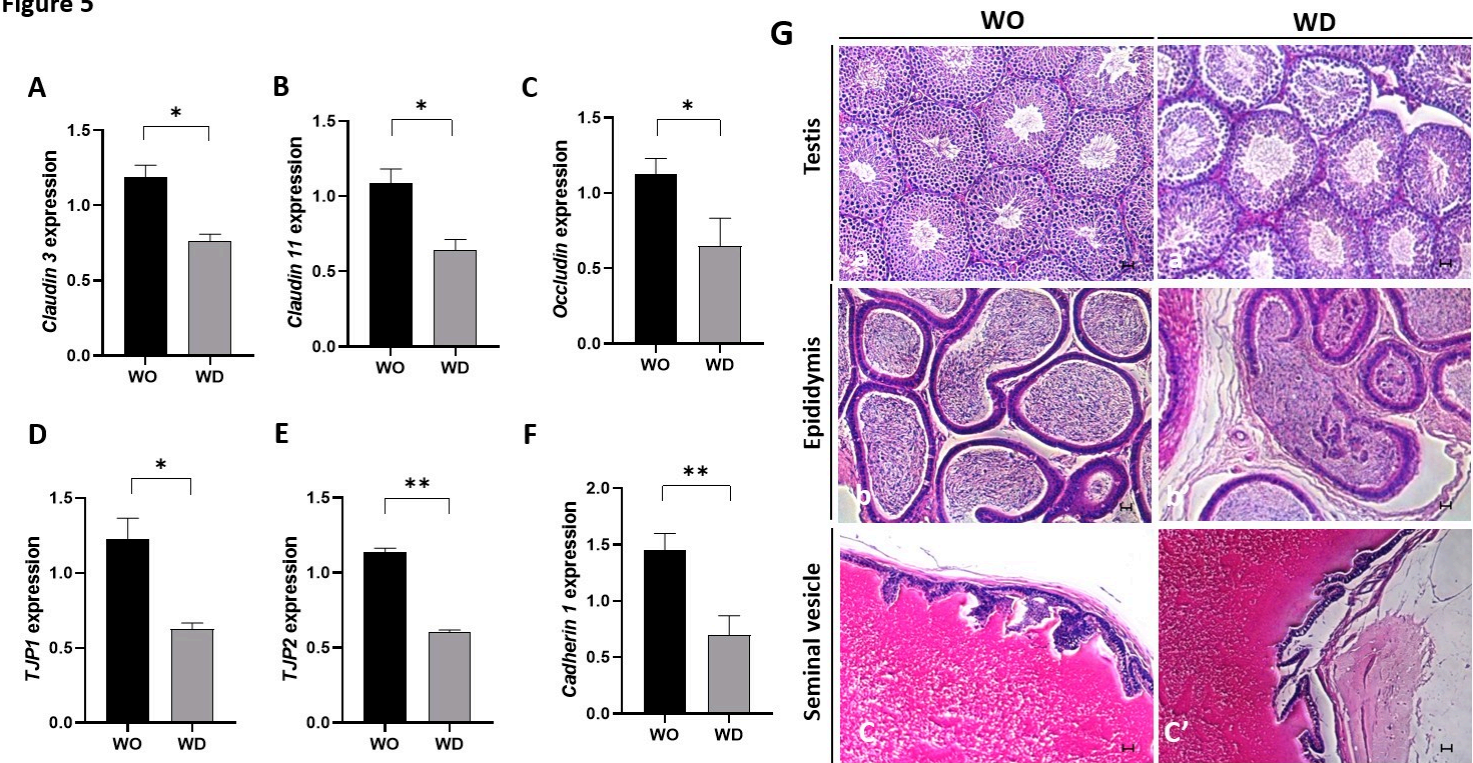


Figure 5

The relative mRNA expression of tight junction proteins in the testis from WO (control) and WD (dysbiosis-induced) mice: Claudin 3 (A), Claudin 11 (B), Occludin (C), tight junction protein (TJP 1, D), TJP 2 (E), and Cadherin 1 (F). (G) Representative Hematoxylin and Eosin (H&E)-stained histological images of the testis, epididymis, and seminal vesicle tissues showing structural differences between the WO and WD groups. Values are expressed as the mean $\pm$ SEM. The superscripts denote significance at * $P < 0.05$ and ** $P < 0.01$.

Figure 6

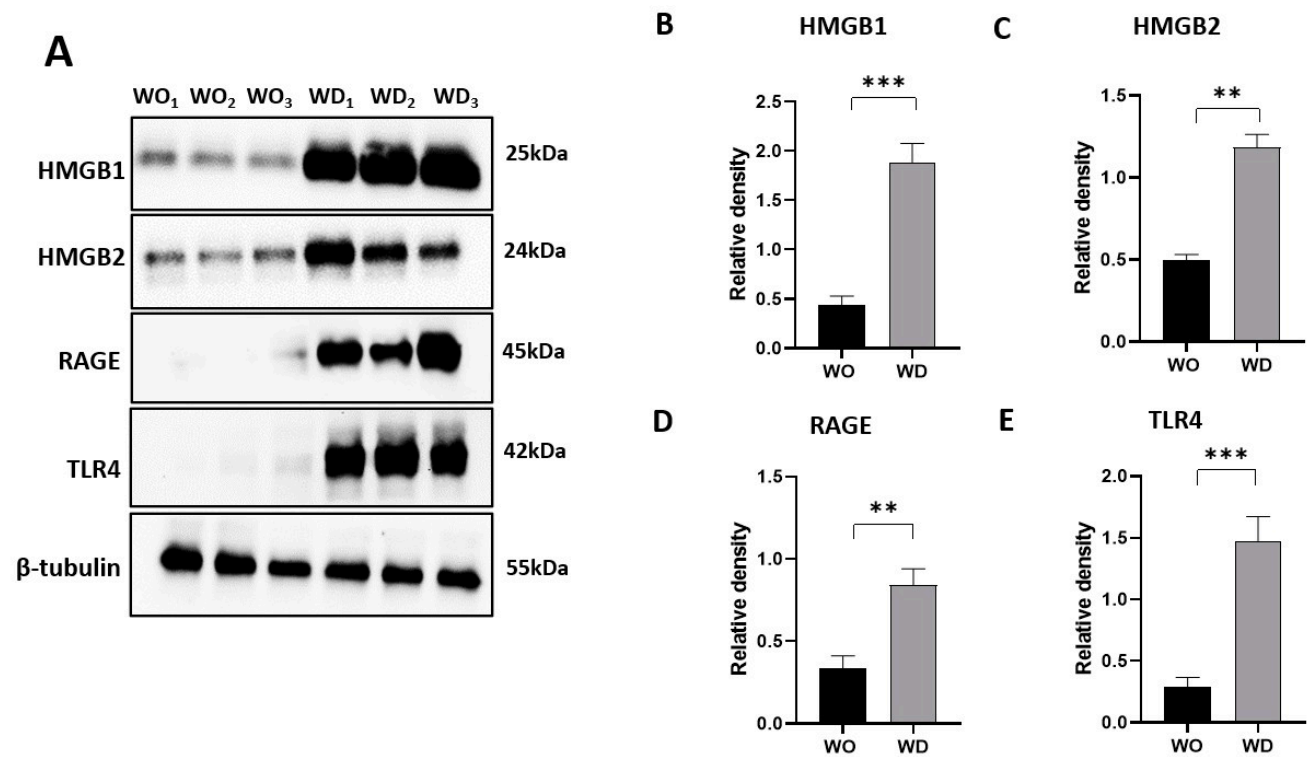


Figure 6

Western blot analysis of high-mobility group box (HMGB)1, HMGB2, receptor for advanced glycation end products (RAGE), and toll-like receptor 4 (TLR4) expression in the testis of dysbiosis-induced (WD) and control (WO) male mice with β -tubulin as a loading control (A). Quantification of relative protein densities for HMGB1 (B), HMGB2 (C), RAGE (D), and TLR4 (E), normalized to β -tubulin density. Values are expressed as the mean $\pm$ SEM. The superscripts denote significance at *P < 0.05 and **P < 0.01. Representative cropped western blot images are presented, and the corresponding full-length uncropped blot images are provided in the Supplementary Information.

Figure 7A

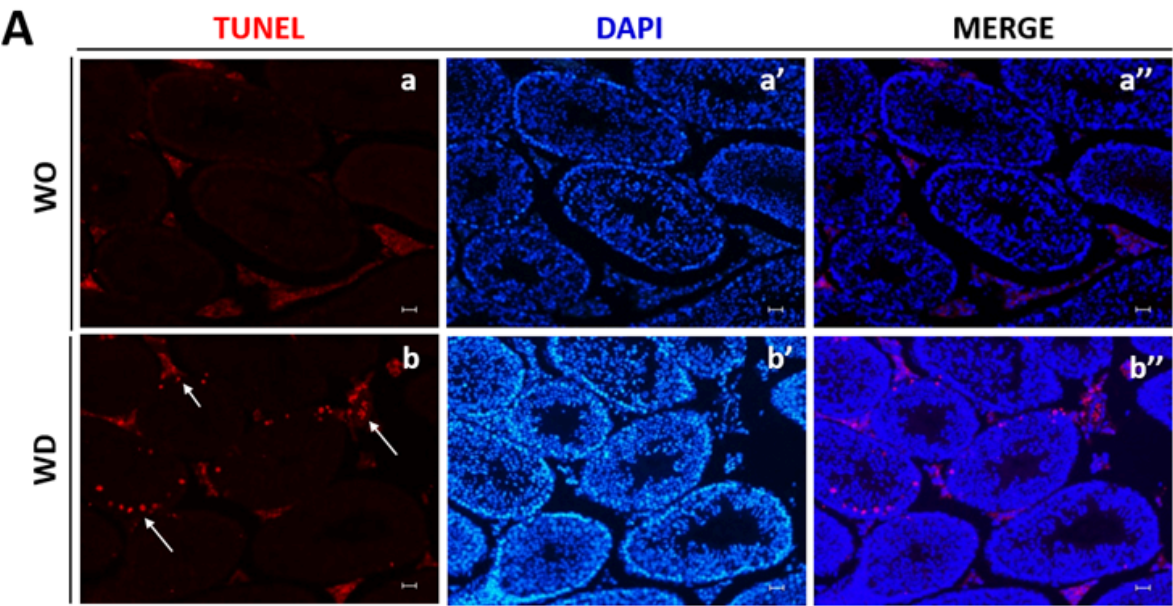


Figure 7B

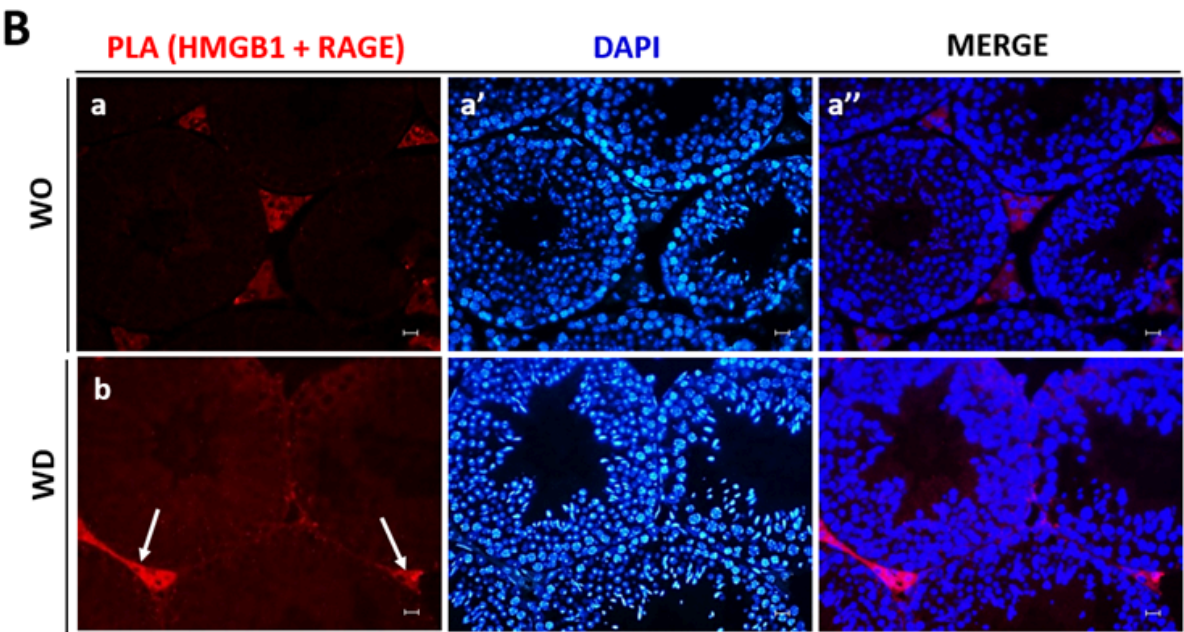


Figure 7

In situ detection of apoptosis (Terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL] assay; A) and high-mobility group box 1-receptor for advanced glycation end products (HMGB1-RAGE) interaction (Proximity ligation assay [PLA]; B) in the testis section of the control (WO) and dysbiosis-induced mice (WD). Representative fluorescence images showing apoptotic cells (white arrows pointing to red fluorescence in b of A) in WD and nuclei stained with 4',6-diamidino-2-phenylindole (DAPI, blue; a' &

b' of A) in testicular sections. Merged images display the spatial distribution of apoptotic signals within the seminiferous tubules (a" &b" of A). (B) Proximity ligation assay (PLA) images showing HMGB1-RAGE interaction (white arrows pointing to red fluorescence in b of B) in the testicular tissue section, with nuclei counterstained by DAPI (blue; a'& b' of B). Merged images indicate the localization of HMGB1-RAGE complexes in the seminiferous epithelium (a" &b" of B).

Supplementary Files

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