Environmental Toxicology and Pharmacology 104 (2023) 104300

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Bisphenol S dysregulates thyroid hormone homeostasis; Testicular survival, redox and metabolic status: Ameliorative actions of melatonin

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ARTICLE INFO

Keywords:
BPS/Melatonin
Testes
TR-α/Dio-2
SIRT1/PGC-1α/Nrf2
p-JAK2/p-STAT3
IR/pAkt/GLUT-1

ABSTRACT

Bisphenol S (BPS) is an incipient threat for reproductive health augmenting societal burden of infertility worldwide. In the present study, we investigated the mechanism of BPS induced testicular dysfunctions and protective actions of melatonin in mice. BPS (150 mg/kg BW) treatment reduced serum T3/T4, testosterone and elevated insulin levels along with adverse effect on thyroid and testicular histoarchitecture. Further, BPS treatment compromised sperm quality, reduced mRNA expression of steroidogenic (StAR/CYP11A1) markers, elevated oxidative load and disrupts metabolic status. However, melatonin (5 mg/kg BW) administration to BPS treated mice showed improved hormonal/histological parameters, enhanced thyroid hormone (TR-α/Dio-2)/melatonin (MT-1) receptor expressions. Further, melatonin treatment modulated the expression of testicular survival/redox (SIRT1/PGC-1α/FOXO-1, Nrf2/HO-1, p-JAK2/p-STAT3), proliferative (PCNA) and metabolic (IR/pAKT/GLUT-1) markers. Furthermore, melatonin treatment enhanced testicular antioxidant status and reduced caspase-3 expression. In conclusion, our results showed that BPS induces endocrine/oxidative and metabolic anomalies while melatonin improved male reproductive health.

1. Introduction

Infertility continues to be a global issue, factors responsible for male infertility/subfertility are diverse and largely unexplored (Inhorn and Patrizio, 2014; Agarwal et al., 2021). Bisphenols are ubiquitous environmental toxins known to alter intracellular redox machinery and are recognized as endocrine disruptors (Adegoke et al., 2020; Manzoor et al., 2022). Bisphenol S (4, 4′-sulfonyldiphenol or BPS) is a relatively new substitute for Bisphenol A (BPA) that has been used in the production of various daily use products (Mukhopadhyay et al., 2022). Evidences from several studies have shown that BPS exposure causes hepatoxicity, nephrotoxicity, neurotoxicity, genotoxicity, metabolic alterations, endocrine disruption and reproductive impairments (Brulport et al., 2021; Huang et al., 2019; Kumar et al., 2020; Pal et al., 2023; Rochester and Bolden, 2015). BPS has been found in human blood, urine, placenta and breast milk at various concentrations (Morimoto et al., 2022).

The growth and functioning of the gonads are significantly influenced by thyroid hormones. Thyroid hormones along with gonadal steroids regulates proliferation and differentiation of testicular cells, spermatogenesis and steroidogenesis (Rashighi and Harris, 2017). Testes expresses thyroid hormone receptors (TR-α/β) and deiodinase-2 (Dio-2), regulating testicular development and functions (Rashighi and Harris, 2017). Structure-based research has demonstrated that BPS alters thyroid hormone receptor (TRα) (Beg and Sheikh, 2020; Siracusa et al., 2018; Zhang et al., 2018). Testes being metabolically active tissue, requires continuous energy fuel to sustain daily production of millions of spermatozoa. Thyroid hormone regulates testicular metabolic homeostasis by regulating glucose uptake and lactate production that nurtures developing germ cells (Wagner et al., 2009; Galardo et al., 2014; Maran, 2003). Besides thyroid hormone, insulin signalling plays a vital role in testicular glucose uptake, germ cell proliferation, hormone synthesis and maintenance of male reproductive health (Meroni et al., 2019). However, investigations regarding BPS-induced altered thyroid hormone and metabolic status are meagre.

Oxidative stress accounts for the leading cause of various health issues (Bisht et al., 2017). Free radical generation results in mitochondrial dysfunction, endoplasmic reticulum (ER) stress, eliciting inflammation

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https://doi.org/10.1016/j.etap.2023.104300
Received 14 June 2023; Received in revised form 30 September 2023; Accepted 18 October 2023
Available online 20 October 2023

and apoptosis, culminating in male infertility. Multiple spectral approaches and molecular docking studies revealed that BPS alters the antioxidant enzyme functions (Zhang et al., 2016). Sirtuin 1 (SIRT1) is a deacetylase known for its ability to regulate a wide range of cellular processes (Khawar et al., 2022; Chianese et al., 2018; Yuan et al., 2016). SIRT1 by deacetylating peroxisome proliferator-activated receptor coactivator 1 alpha (PGC1α), upregulated glucose metabolism and lactate synthesis in the testes via Insulin (IR/PI3K/AKT/GLUT-1) signalling pathway (Ren et al., 2020). SIRT1 activates transcription factors forkhead box O-1 (FOXO-1) and nuclear factor E2–related factor-2 (Nrf2), which upregulates the expression of antioxidant enzyme genes and prevents germ cell apoptosis (Kumar et al., 2020). Aside from that, Sue et al. (2013) documented that SIRT1 and PGC1α work together to enhance T3 response, which eventually regulates testicular development and maturation (Suh et al., 2013). However, the molecular insights of BPS induced testicular oxidative stress are still unclear.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway regulates a variety of biological processes such as inflammation, proliferation, tissue repair and apoptosis (Dodington et al., 2018; Hu et al., 2021; Xin et al., 2020). There are four members of JAK family and six members of STAT family out of which JAK2 and STAT3 is mainly expressed in testes (Hu, 2021; Lan et al., 2019; Wu et al., 2022). Activated STAT3 dimer suppressed the production of the apoptotic markers BAX, caspase-3, and NFκB signalling and decreased oxidative stress, which led to cell survival (He et al., 2022). But the reports regarding effect of BPS exposure on testicular JAK2/STAT3 expressions are largely unexplored.

Melatonin is a potent antioxidant, anti-inflammatory, anti-apoptotic molecule (Ferlazzo et al., 2020; Hansda and Haldar, 2021; Hyun et al., 2023; Kvetnoy et al., 2022), directly scavenge free radicals as well as indirectly promotes transcription of various antioxidant enzymes (Ferlazzo et al., 2020). Local testicular melatonin synthesis and presence of its receptor (MT-1) further imply its significance in regulation of male reproduction (Kumar et al., 2021; Mukherjee and Haldar, 2014). Recent reports suggests that melatonin modulates SIRT-1 signalling pathway and proved to be protective molecule against hepatotoxicity, nephrotoxicity, reproductive toxicity and immune regulation (Deng et al., 2020; Kumar et al., 2020; Pal et al., 2023; Song et al., 2019). Therefore, the present study aims to investigate BPS-induced thyroid dysfunction, molecular determinates of testicular redox/metabolic stress and the ameliorative actions of melatonin.

2. Materials and methods

2.1. Animal care and maintenance

Adult male Parkes (P) strain mice of 90 days were used in the present experiment and were kept in departmental animal house facility (1802/GO/RE-Bi/S/15/CPCSEA dated 25–02–2015) of Banaras Hindu University, Varanasi, India. They were kept in polypropylene cages under regular light/dark cycle (12 L: 12D), temperature (25 ± 2 °C) and were provided with commercial food pellet and drinking water ad libitum. All experiments were performed in accordance with the guidelines of Institutional Animal Ethical Committee (IAEC) of Banaras Hindu University (IAEC reference number-BHU/DoZ/IAEC/2018–2019/036) and Committee for the Purpose of Supervision and Control on Experiments on Animals (CPSCEA), Government of India.

2.2. Chemicals and reagents

BPS (4, 4' –Sulfonyldiphenol, CAS Number: 80–09–1) was bought from Sigma-Aldrich Chemicals, St. Louis, MO. Melatonin (CAS #73–31–4 product code- 70902) was purchased from SRL India. The nitrocellulose membrane (Cat # 162–0115, 0.45 μm) and chemiluminescence ECL kit (Cat # 170–50 600) were procured from Bio-Rad Laboratories, Inc. made in Germany. ELISA kits were acquired from Diametra Spello-PG, Italy, Cal biotech. Other chemicals utilized in the present study were bought from Hi Media, SRL, and Merck, India.

2.3. Experimental design and sample collection

Adult male mice (90 days) were randomly allocated to four (I-IV) groups (n = 5/group) and treated as follows:

Group I: Control (corn oil as vehicle).

Group II: Melatonin treated (5 mg/kg BW/alternate day; i.p. injection).

Group III: BPS treated (150 mg/kg BW/day; orally).

Group IV: BPS plus melatonin treated.

Vehicle, BPS were administered daily with the help of oral feeding gavage, while intraperitoneal injections of melatonin were administered between 10:30 am and 11:30 am at every alternate days for 28 days. After 24 h of the last treatment, animals were weighed and sacrificed. Blood was collected from the trunk region; serum was separated and stored at − 20 °C for further analyses. Testes of the left side and thyroid gland were excised from the animals in each group, blotted dry, weighed, and then quickly immersed in Bouin's fixative for histological, detection of testicular cell apoptosis by Terminal 2-deoxyuridine 5'-triphosphate nick end-labelling (TUNEL) assay and immunohistochemical studies, while right testes were immediately kept in − 20 °C for biochemical, quantitative real-time PCR (qRT-PCR) and western blot analyses. Cauda epididymis of either sides from the animals in each group were used for assessment of various sperm parameters.

2.4. Peripheral hormone analyses

The circulatory levels of testosterone (DiaMetra, Italy-DKO002), T3 (Calbiotech T3379T), T4 (Calbiotech T4224T), and insulin (DiaMetra, Italy – DKO076) were measured in serum by using commercial ELISA kits with protocols supplied with the kit.

2.5. Sperm analyses (motility, count, viability and morphology)

Cauda epididymis from each groups were minced in 0.5 ml complete medium to prepare sperm suspension. Sperm motility was evaluated by counting at least 200 motile and non-motile spermatozoa by taking a drop of sperm suspension on a clean slide and observed under 40X magnification (WHO, 1999). The suspension was further diluted ten times and then used for counting the spermatozoa by taking a drop of the suspension onto the Neubauer's slide of the Hemocytometer. Spermatozoa were counted in all the four WBC chambers of the Neubauer's slide. The sperm count was recorded by the unit of 10^6/ml (Kumar et al., 2019). Sperm viability was assessed via MTT Assay, briefly, after the sperm count, the sperm suspension was diluted to 4 million spermatozoa/ml; from this 100 μl of suspension and 20 μl of MTT (stock solution 5 mg/ml) were placed in a 96-well plate. Then, the plate was incubated at 37 °C with 5% CO2 for 1 h. After that, 200 μl of acidified propanol was added in each well to dissolve the formazan. After 30 min, optical density was recorded on a micro-plate reader (ELx-800; Biotek Instruments, Winooski VT, USA) at a wavelength of 570 nm (Singh and Singh, 2019). Sperm morphology was analysed through eosin-nigrosin staining method.

2.6. Histological and histomorphometric analyses-

Testes and thyroid gland from each group were fixed in Bouin's fixative for 24 h at room temperature, dehydrated in different grades of ethanol, cleared in xylene and embedded in paraffin wax. Sections were cut at 7 μm with a microtome (RM2245 semi-automatic microtome, Leica, Germany), spreaded on 2% gelatin pre-coated slides and then stained with haematoxylin and eosin. The stained sections were then observed under a light microscope (Nikon, E 200, Tokyo, Japan) and histomorphometry was carried with inbuilt software (NIS-Element D)

Table 1
Primer sequences of various markers used for qPCR analyses-.

Gene	Forward Primer	Reverse Primer	Accession No.
StAR	GAAAGCCAGCAGGAGAACG	GCGGTCCACAAGTTCTTCAT	>NM_011485.5
CYP11A1	AACCTTTCCTGAGCCCTACG	TAGCCAACCATTGTCGCCAG	>NM_019779.4
MT-1	TCTACACTGGCCTTCATCCTCA	GCCACAGCTAAACTCACCACA	>NM_008639.3
SIRT-1	CTCTAGTGACTGGACTCCGC	AACAATCTGCCACAGCGTCA	>NM_019812.3
PGC-1α	GGGGCACATCTGTTCTTCCA	GCTTGACAGGGATGACCGAA	>NM_008904.3
FOXO-1	TCAACCTTCTCTCGTCCCCA	ATGCTGGATTGGCCGTATGT	>NM_019739.3
Nrf2	CAGTGCTCCTATGCGTGAA	GCGGCTTGAATGTTTGTCT	>NM_010902.5
HO-1	TGACAGAAGAGGCTAAGACCG	AGTGAGGACCCACTGGAGGA	>NM_010442.2
B-actin	GATATCGCTGCGCTGGTCG	CATTCCCACCATCACACCCT	>NM_007393.5

according to published method (Hansda and Haldar, 2021; Kumar et al., 2021; Pal et al., 2022a).

2.7. Biochemical estimation

Testes homogenate (10%w/v) was prepared in ice-cold PBS (150 mmol, pH 7.4). Bradford method was used to estimate protein concentration of the samples (Pal et al., 2022a). Estimation of superoxide dismutase (SOD) activity was performed following the spectrophotometric method (Kumar et al., 2019). Catalase (CAT) activity was measured following an indirect spectrophotometric method (Kumar et al., 2019). Lipid peroxidation (LPO) was measured by estimation of malondialdehyde (MDA) level following the method published elsewhere (Pal et al., 2022c). The nitric oxide (NO) level was determined by direct spectrophotometric method following the protocol as published previously (Hansda and Haldar, 2021; Kumar et al., 2021; Pal et al., 2023). Testicular glucose was estimated using a commercially available kit (Auto Span Diagnostics Ltd., Mumbai India) by a quantitative colorimetric method. Testicular ROS content was measured by estimating the relative fluorescence intensity of DCF by DCFDA (2'−7'-Dichlorodihydrofluorescein diacetate) dye method. Briefly, 100 µl of freshly prepared testicular homogenate was mixed with 200 µl of PBS (150 mM, pH 7.4) followed by incubation with 10 µM DCFDA for 30 min in dark. Cellular esterase cleaves the diacetate group, and intracellular ROS oxidises the non-fluorescent DCHF-DA to produce the highly fluorescent product 2, 7-dichlorodihydrofluorescein (DCF) having excitation and emission range at 485 nm and 535 nm respectively. Result was expressed as relative fluorescence intensity per mg protein (RFI/mg protein).

2.8. qRT-PCR analyses

Total RNA from testes was isolated using the TRIZOL reagent and cDNA was synthesized using First Strand cDNA Synthesis Kit (catalog no. #K1622, Thermo Fisher Scientific). Gene-specific primers (Table 1) of steroidogenic and redox markers were used for qPCR amplification with the synthesised cDNA as template. The internal control β-actin gene was used to normalize the quantification of mRNA target. qRT-PCR was performed using the ABI7500 instrument at the Interdisciplinary School of Life Science (ISLS), Banaras Hindu University with SYBR Green Master Mix (Maxima SYBR Green/ROX qPCR Master Mix 2X #K0221). Data were analyzed by comparative ΔΔCt method.

2.9. Immunohistochemical analyses

The immunohistochemical assessment of proliferation cell nuclear antigen (PCNA) was accomplished using protocol published elsewhere (Pal et al., 2022b). Briefly, 7 µm thick stretched sections of testes were first deparaffinised and then sequentially rehydrated. Slides were treated with 0.1% H2O2 in methanol for 30 min at room temperature to impede the endogenous peroxidase activity. Then the slides were incubated with horse blocking serum (1:100 in PBS; Vectastain ABC Universal Kit; PK-6200, Vector Laboratories) for 1 h at room temperature to block the nonspecific binding. The slides were then kept at 4 °C in a humidified chamber incubated with the primary antibody against PCNA (SC 7907, dilution 1:100) overnight. The next day, slides were incubated with biotinylated secondary antibody (dilution 1:50; Vectastain ABC Universal Kit; PK-6200, Vector Laboratories) at room temperature for 2 h and then conjugated with a preformed ABC for 30 min. The immunoreactivity was observed using 0.03% peroxidase substrate 3, 3–diaminobenzidine (DAB; ImmPACT™ DAB, SK-4105, Vector Laboratories). Sections were then serially dehydrated, mounted with DPX, and observed under Nikon Eclipse E200 microscope with NIS-Element D software.

Table 2
Details of different antibodies used for western blot analyses:.

Antibody	Host species	Dilution	Catalogue no.	Source
TRα	Rabbit	1:500	SAB4502968	Sigma-Aldrich
Dio-2	Rabbit	1:500	ab77779	Abcam
MT-1	Goat	1:500	SC-13186	Santa-Cruz biotechnology
SIRT-1	Rabbit	1:500	ab189494	Abcam
pJAK-2	Rabbit	1:500	D15E2	Cell Signalling
pSTAT-3	Rabbit	1:250	D3A7	Technology Cell Signalling Technology
IR	Rabbit	1:250	PAA895Mu01	Cloud clone
GLUT-1	Rabbit	1:500	PA1–46152	Thermo scientific
p-AKT	Rabbit	1:250	9271	Cell signalling technology
Caspase-3	Goat	1:500	SC-7148	Santa-Cruz biotechnology
β-actin	Rabbit	1:1000	4970 T	Cell signalling technology
Secondary:				
Rabbit IgG	Goat	1:2000	32067	GeNei
Goat IgG	Rabbit	1:3000	114048001 A	GeNei

2.10. Western blot analyses

Western blot analyses was performed as per previously published paper (Kumar et al., 2019). Briefly, 10%w/v testicular homogenate was prepared in RIPA buffer containing mixture of various protease inhibitors, then centrifuged at 12,000 g for 30 min, at 4 °C and the protein content of the supernatant was estimated by Bradford assay. 60 µg protein was then resolved on SDS-PAGE, followed by electro transfer (Trans-blot Turbo Bio-Rad) on nitrocellulose membrane for 10 min and then incubated with primary antibodies (details given in Table 2) overnight. Immunodetection was done using HRP-tagged specific secondary antibodies and then visualization using ECL Kit. The relative band intensity was measured by Alpha Imager 2200 software (Alpha Innotech) with respect to β-actin (internal loading control).

2.11. TUNEL assay

TUNEL (Terminal 2-deoxyuridine 5'-triphosphate nick end-labeling) assay was performed in paraffin embedded testicular tissue sections of 7

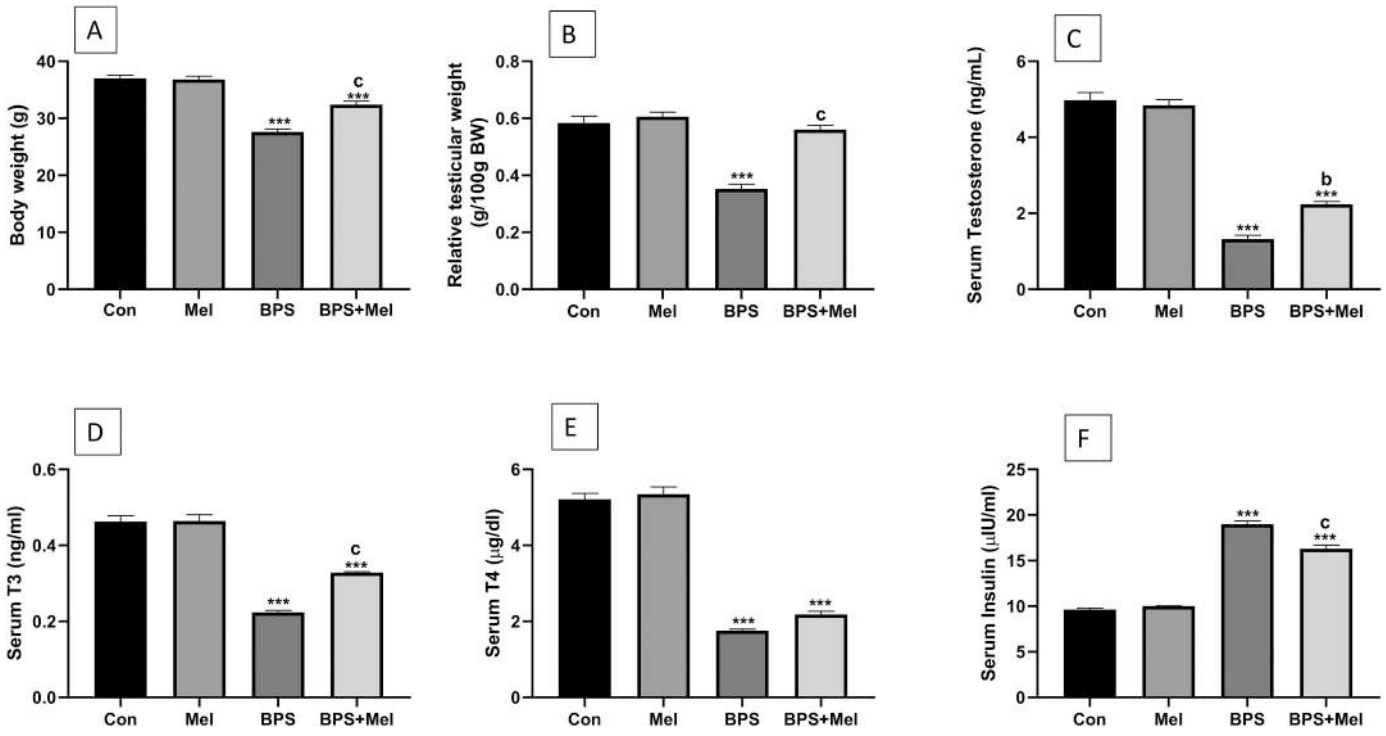

Fig. 1. Effect of BPS and melatonin treatment on weight and circulatory hormonal levels (A) body weight, (B) relative testicular weight (C) serum testosterone, (D) serum T3, (E) serum T4, (F) serum insulin levels in adult male mice. Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

µm using One-step TUNEL *In Situ* Apoptosis Kit Green, Elab Fluor®488 (Cat. No. E-CK-A321; Elabscience), as per the manufacturer's instruction. The slides were analysed with Laser Scanning Super Resolution Microscope System (SP8 STED, Leica Microsystems, Germany) with Leica-LAS X software. DNA strand breaks, represented by TUNEL positive nuclei, was assessed and were counted using 40X magnification images.

2.12. Statistical analyses

Statistical analyses of data was done by Graph Pad Prism 8 software (USA) with one-way ANOVA followed by Tukey's multiple range tests for multiple comparisons. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of BPS and Melatonin treatment on weight parameters

A significant ($p < 0.001$) decrease in body weight and relative testicular weight was observed in BPS only treated animals when compared with control. In contrast, melatonin supplementation to BPS significantly ($p < 0.001$) restored weight parameters when compared with BPS only treated mice (Fig. 1A and B).

3.2. Effect of BPS and Melatonin treatment on hormonal levels

Marked alterations were detected in circulatory testosterone, thyroid hormone (T3 and T4), and insulin levels in different experimental groups. A significant ($p < 0.001$) decline in the circulatory testosterone, thyroid hormone (T3 and T4), and a significant increase in circulatory insulin levels were observed in BPS only treated group when compared with control. On the contrary, BPS plus melatonin treated group exhibited a significant increase in the levels of testosterone ($p < 0.01$), serum T3 ($p < 0.001$) while significant decrease in insulin ($p < 0.001$) levels when compared with BPS alone treated animals (Fig. 1C-F).

3.3. Effect of BPS and Melatonin treatment on sperm parameters

A significant decline ($p < 0.001$) in %motile sperm, sperm count, viability, %normal sperm were observed in BPS only treated group when compared with control. On the other hand, BPS plus melatonin treated animals significantly ($p < 0.001$) restored the sperm quality when compared with BPS only treated animals (Fig. 2A-D).

3.4. Effect of BPS and Melatonin treatment on testicular histoarchitecture

Histoarchitectural assessment of the testes of control and melatonin treated group showed normal appearance of seminiferous tubule as evident by sequentially arranged germ cells, lumen filled with sperm (Fig. 2E-F), while BPS only treated group showed marked testicular degenerative changes as evident by presence of vacuoles, reduced germ cell number, germ cell loosening and lumen devoid of sperm (Fig. 2G). In contrast, BPS plus melatonin treated group showed restoration of spermatogenesis (Fig. 2H). Further, testicular morphometric analyses showed a significant ($p < 0.001$) decrease in height of the germinal epithelium, seminiferous tubule diameter and area in BPS only treated group as compared to the control. BPS plus melatonin treated group showed a significant ($p < 0.001$) increase in testicular morphometric parameters as compared to BPS alone treated group (Fig. 2I-K).

3.5. Effect of BPS and Melatonin treatment on histoarchitecture of thyroid gland

The thyroid gland histoarchitecture of control and melatonin treated group showed numerous follicles of different sizes, the follicles were lined by epithelial cells (Fig. 3A-B), while BPS only treated group showed disordered thyroid histoarchirtecture as evident by thinning of follicular epithelial cells and vacuolated follicular epithelium (Fig. 3C). In contrast, BPS plus melatonin treated groups showed improved

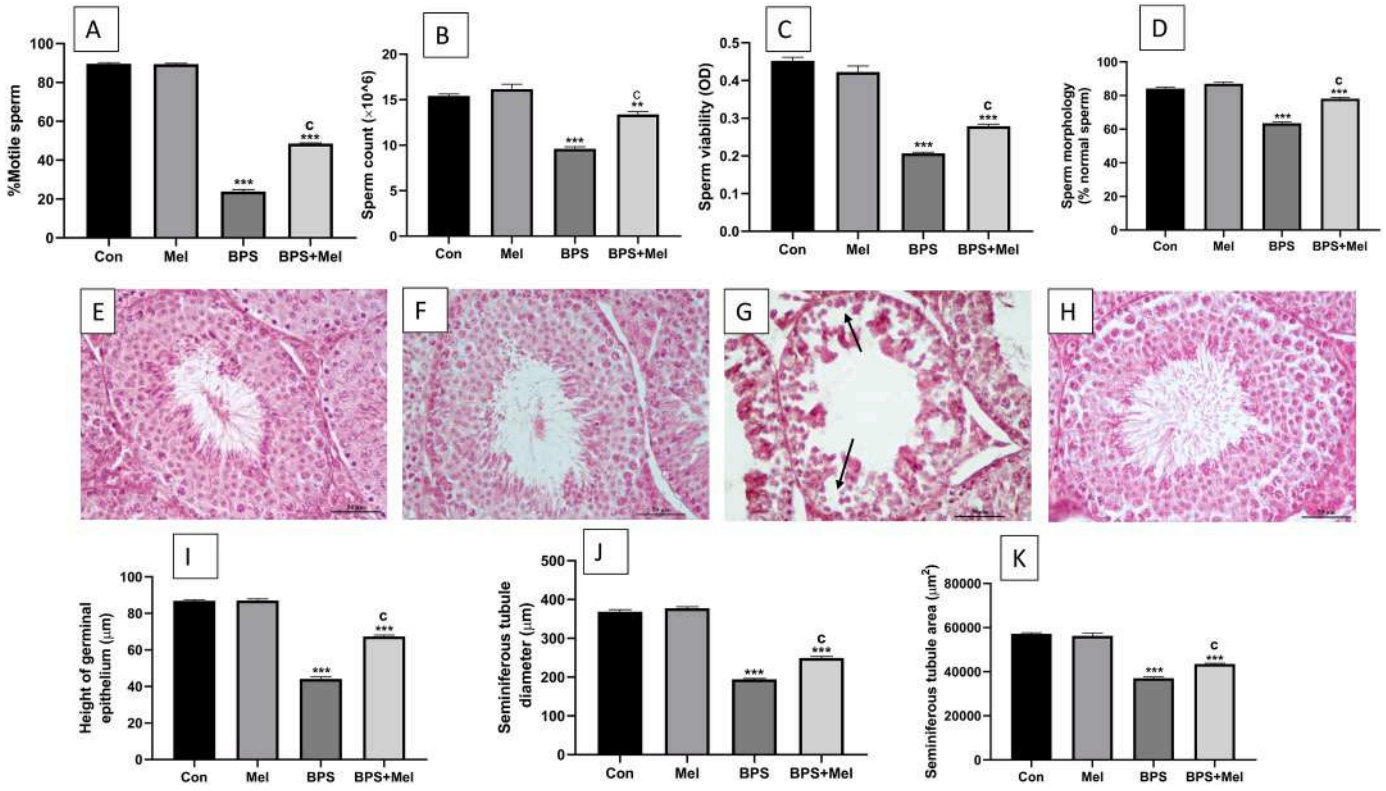

Fig. 2. Effect of BPS and melatonin treatment on sperm parameters and testicular histoarchitecture in adult male mice following Hematoxylin and Eosin staining. (A-D) showing %motile sperm, sperm count, sperm viability and sperm morphology respectively. Photomicrographs (E and F) showing normal spermatogenesis process within the seminiferous tubule with sequentially arranged germ cells and lumen filled with spermatozoa in control and melatonin treated group respectively. (G) BPS treated group showing testicular degeneration with loosening of germ cells, vacuolisation, lumen devoid of spermatozoa, blank cavities (indicated by arrow). (H) BPS plus melatonin treated group showing improved testicular histoarchitecture. Original magnification (E-H) at × 400. (I-K) showing height of germinal epithelium, change in diameter of seminiferous tubule, and seminiferous tubule area respectively. Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

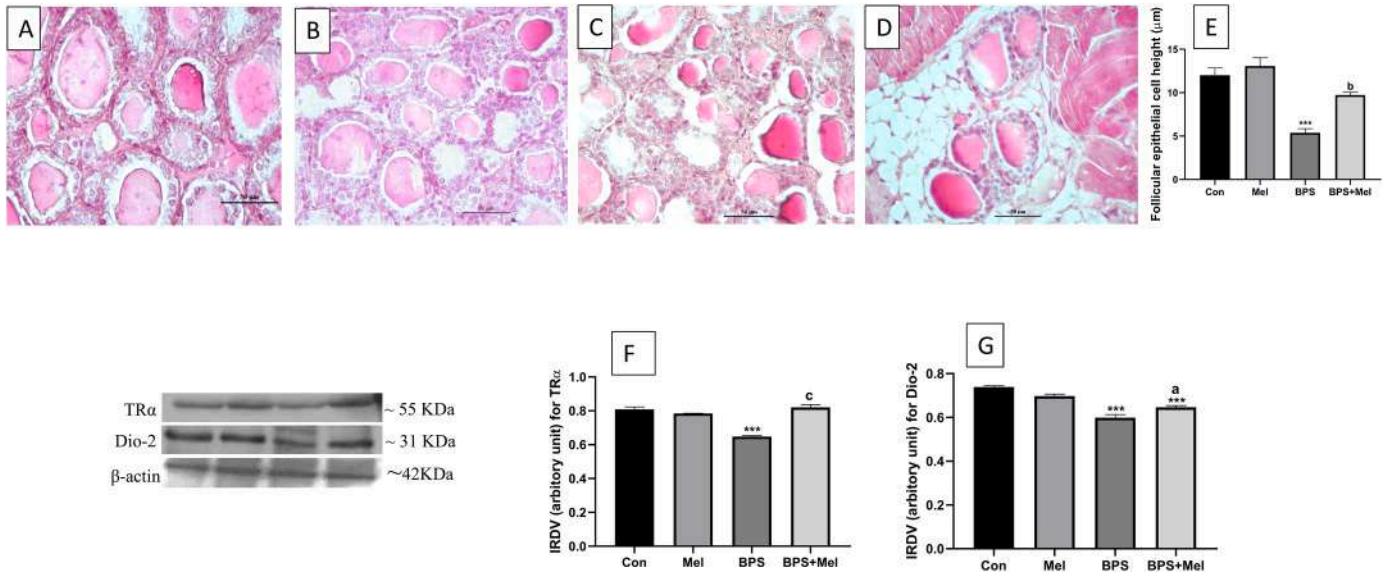

Fig. 3. Effect of BPS and melatonin treatment on histoarchitecture of thyroid gland in adult male mice following H & E staining and testicular TR-α and Dio-2 expression. Photomicrographs (A and B) showing thyroid gland in control and melatonin treated group. The thyroid gland showed numerous follicles of different size, that were lined by simple epithelial cells and lumen filled with colloid. Photomicrograph (C) BPS treated group showing thinning of follicular epithelial cells and vacuolated follicular epithelium. (D) Thyroid follicles of BPS plus Melatonin treated group showing most of the follicles regained its normal architecture. Original magnification (A-D) at × 400. (E) showing follicular epithelial cell height. (F and G) showing densitometric analyses testicular TRα and Dio-2 expressions respectively, represented as relative band intensity. β-Actin expression was used as loading control. Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

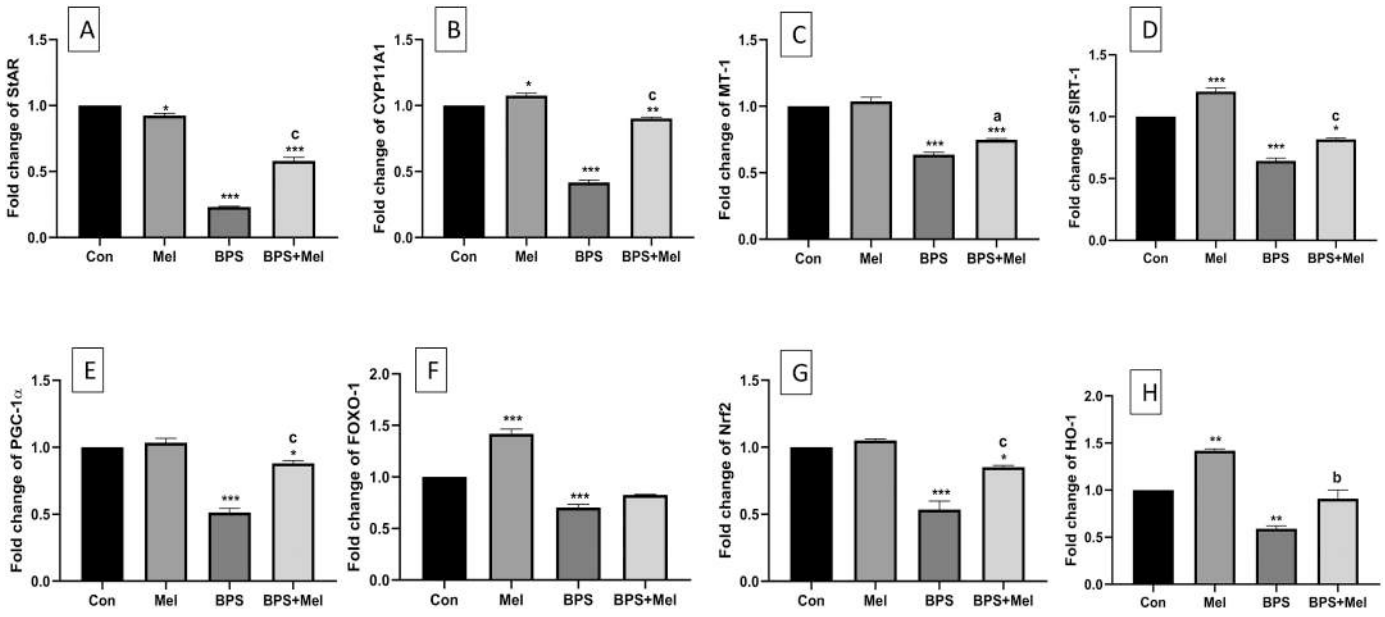

Fig. 4. Effect of BPS and melatonin treatment on mRNA expression of testicular steroidogenic and redox markers. (A-H) showing fold change in the expression of StAR, CYP11A1, MT-1, SIRT-1, PGC-1α, FOXO-1, Nrf2 and HO-1 respectively. Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

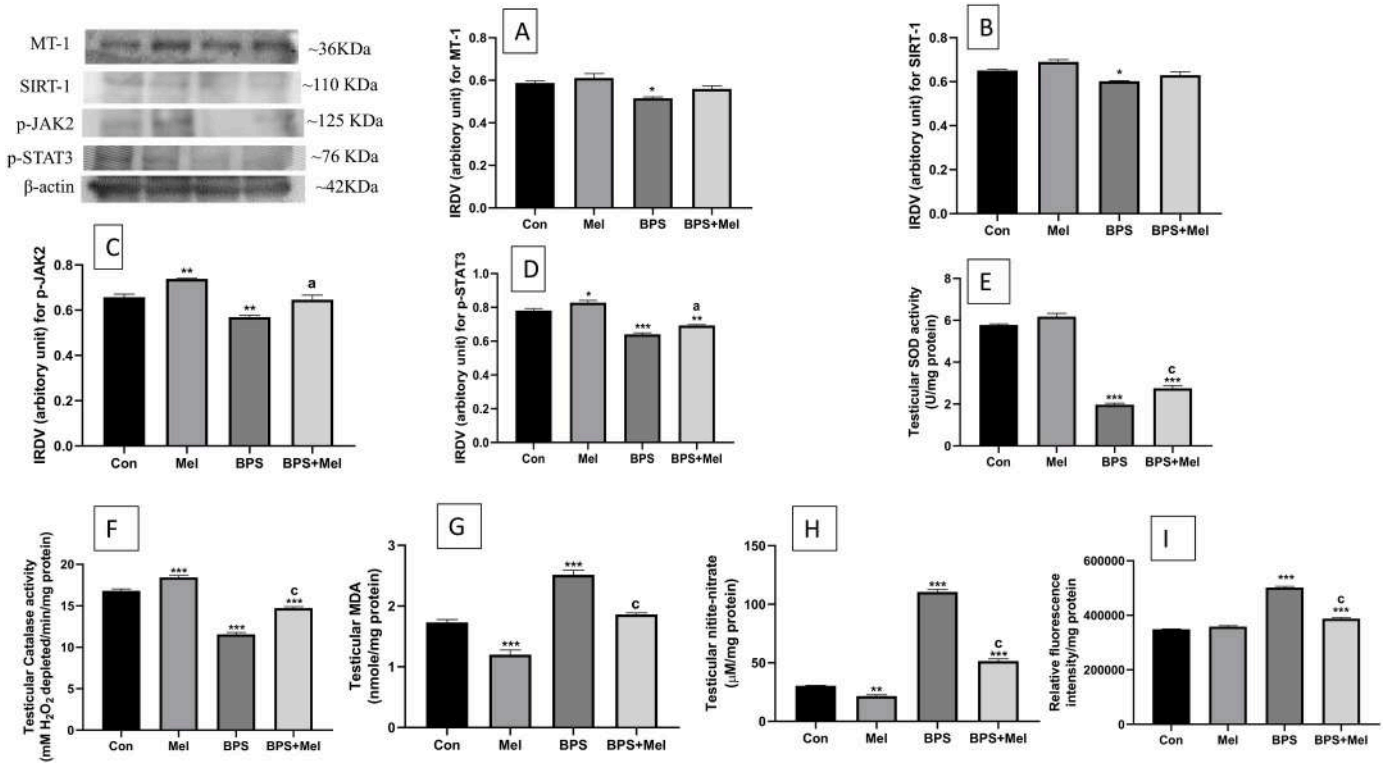

Fig. 5. Effect of BPS and melatonin treatment on testicular redox markers and oxidative load in adult male mice. (A-D) showing densitometric analyses of testicular MT-1, SIRT-1, p-JAK2 and p-STAT3 respectively. β-Actin expression was used as loading control. (E and F) showing testicular SOD and catalase activity respectively. (G and H) showing lipid peroxidation and nitrite-nitrate level respectively, (I) showing testicular relative fluorescence intensity of 2', 7'-dichlorofluorescein (DCF). Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

histoarchitecture (Fig. 3D). Further thyroid morphometric analyses showed a significant reduction in follicular epithelial cell height ($p < 0.001$) in BPS only treated group as compared to the control. BPS plus melatonin treated group showed a significant increase ($p < 0.01$) in follicular epithelial cell height when compared to BPS alone treated group (Fig. 3E).

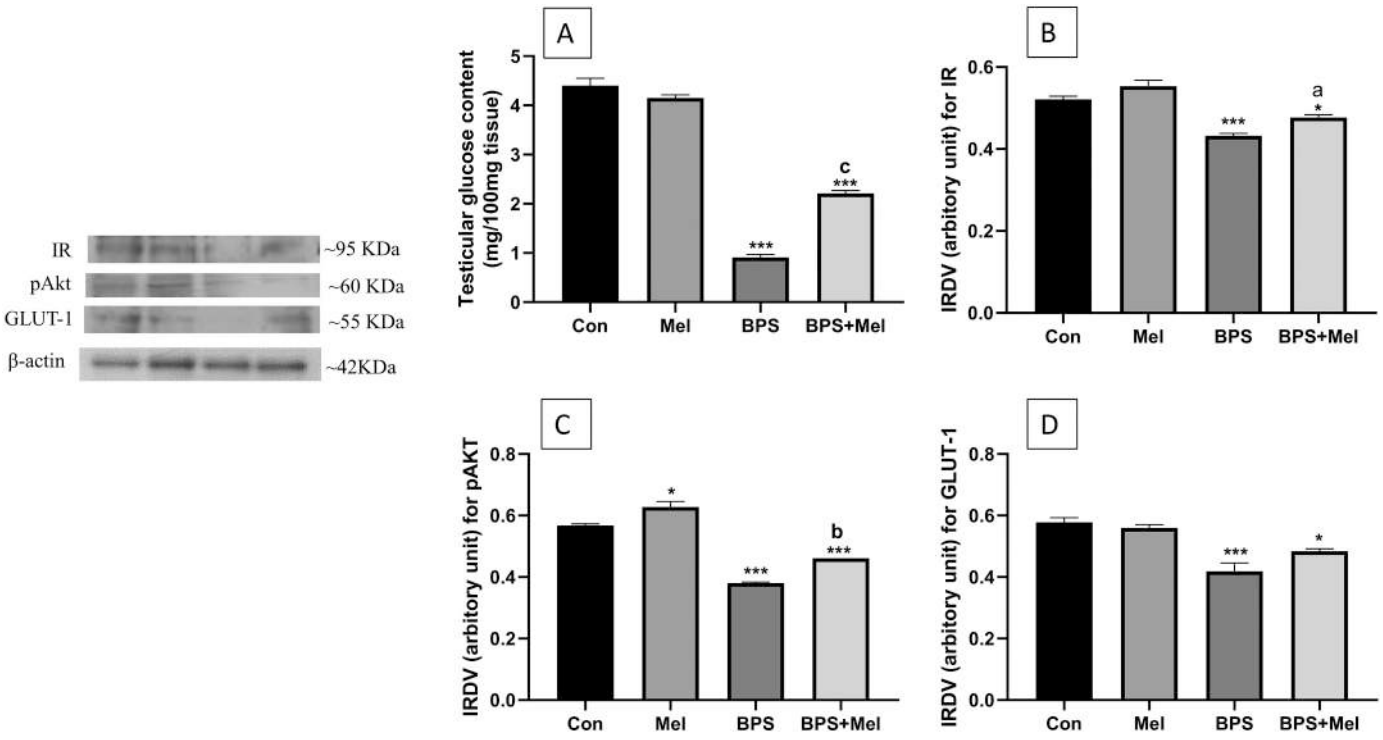

Fig. 6. Effect of BPS and melatonin treatment on testicular metabolic markers. (A) showing testicular glucose level. (B-D) showing densitometric analyses of testicular IR, pAKT and GLUT-1 respectively. β-Actin expression was used as loading control. Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

3.6. Effect of BPS and Melatonin treatment on testicular TR-α and Dio-2 expression

A significant ($p < 0.001$) decrease in testicular TR-α and Dio-2 expressions was observed in BPS only treated group as compared to control while, BPS plus melatonin treatment showed a significant increase in TR-α ($p < 0.001$) and Dio-2 ($p < 0.05$) expressions when compared with BPS alone treated group (Fig. 3F-G).

3.7. Effect of BPS and Melatonin treatment on testicular steroidogenic, survival and redox markers

The qPCR analyses revealed that BPS treatment significantly ($p < 0.001$) decreased mRNA expression of steroidogenic enzymes StAR and CYP11A1 as compared to control; while BPS plus melatonin treated group showed a significant ($p < 0.001$) increase in the mRNA expression of StAR and CYP11A1 when compared with BPS alone treated group

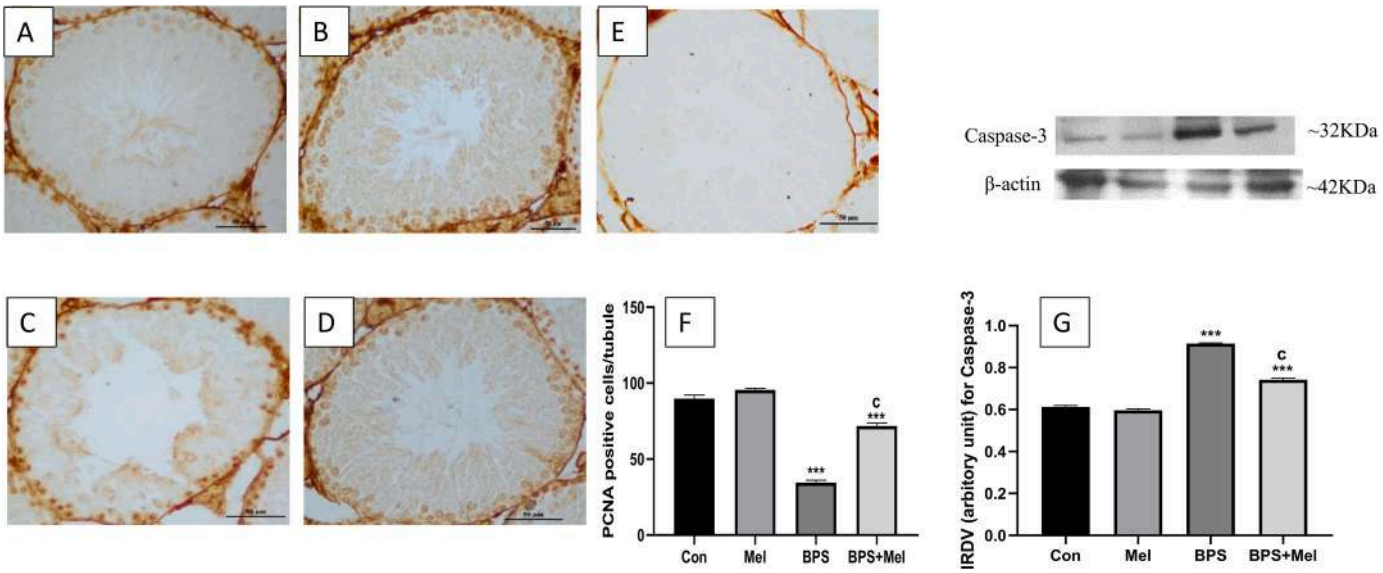

Fig. 7. Effect of BPS and melatonin treatment on testicular cell proliferation and apoptotic markers. Photomicrographs (A-D) showing immunohistochemical localization of PCNA positive cells in (A) control, (B) melatonin, (C) BPS, (D) BPS plus melatonin treated groups, (E) showing negative control (without primary antibody of PCNA). (F) showing PCNA positive cells/tubule. (G) showing densitometric analyses of germ cell apoptotic marker caspase-3. β-Actin expression was used as loading control. Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

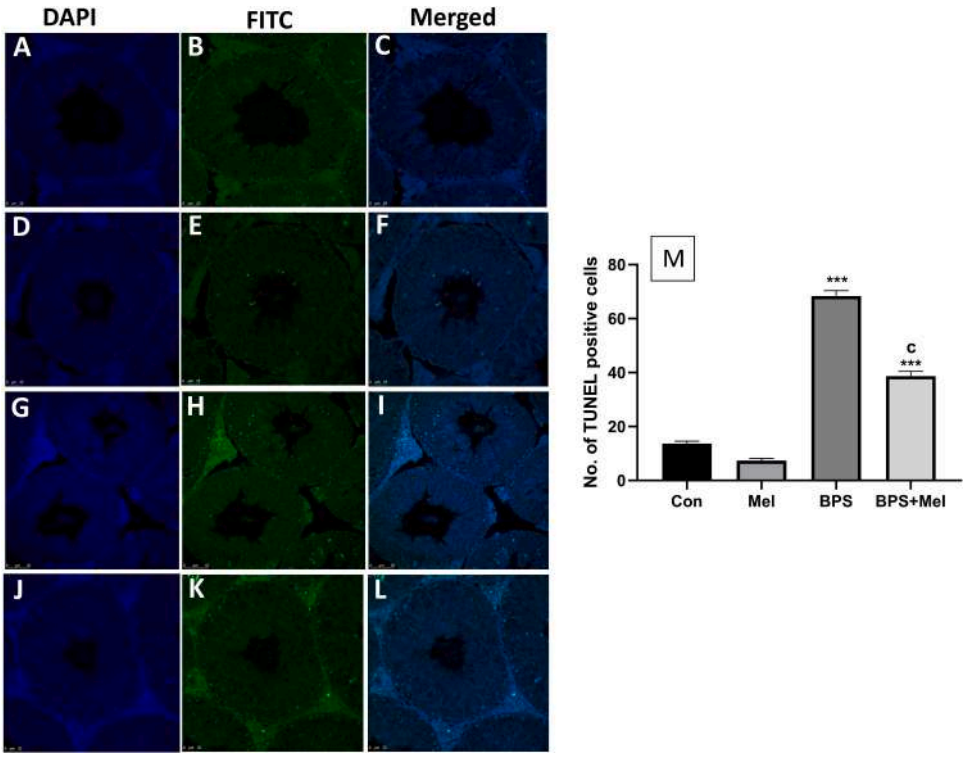

Fig. 8. Effect of BPS and melatonin treatment on apoptosis of testicular cells by TUNEL assay in control (A-C); melatonin (D-F); BPS (G-I); BPS plus melatonin (J-L) treated groups at × 400 magnification. A,D,G,J denotes DAPI staining; B,E,H,K shows FITC staining. C,F,I,L shows merged images. (M) showing number of TUNEL positive cells. Values are expressed as Mean ± SEM. Significance of difference; * ($p < 0.05$) * * ($p < 0.01$) and * ** ($p < 0.001$), control vs other experimental groups; a ($p < 0.05$), b ($p < 0.01$) and c ($p < 0.001$) BPS vs. BPS+Mel.

(Fig. 4 A-B). A significant ($p < 0.001$) reduction in mRNA expressions of MT-1, SIRT-1, PGC-1α, FOXO-1, Nrf2 and HO-1 ($p < 0.01$) was observed in BPS only treated group as compared to control; while BPS plus melatonin treated group showed a significant ($p < 0.001$) increase in the mRNA expression of SIRT-1, PGC-1α, Nrf2, MT-1 ($p < 0.05$) and HO-1 ($p < 0.01$) when compared to BPS only treated group (Fig. 4 C-H). Further western blot analyses revealed, a significant decrease in testicular MT-1 ($p < 0.05$), SIRT1 ($p < 0.05$), p-JAK2 ($p < 0.01$) and p-STAT3 ($p < 0.001$) expressions in BPS treated group as compared to control while, BPS plus melatonin treatment showed a significant increase in p-JAK2 and p-STAT3 ($p < 0.05$) expressions when compared with BPS alone treated group (Fig. 5A-D).

3.8. Effect of BPS and Melatonin treatment on testicular oxidative load

A significant ($p < 0.001$) decrease in testicular antioxidant enzyme (SOD & CAT) activity was observed in BPS only treated animals when compared with control group while, BPS plus melatonin treated group showed a significant ($p < 0.001$) increase in SOD and CAT activities when compared with BPS alone treated group. Further, the level of MDA a marker of lipid peroxidation and testicular nitrite-nitrate was significantly ($p < 0.001$) increased in testes of BPS only treated group as compared to control. BPS plus melatonin treatment significantly ($p < 0.001$) decreased the level of MDA and nitrosative stress when compared with BPS alone treated group (Fig. 5E-H). Furthermore, intracellular ROS content was significantly elevated ($p < 0.001$) in BPS only treated groups as compared to control; while BPS plus melatonin treated group showed a significant ($p < 0.001$) decrease in intracellular ROS level when compared with BPS alone treated group (Fig. 5I).

3.9. Effect of BPS and Melatonin treatment on testicular metabolic markers

A significant decrease in testicular glucose level ($p < 0.001$), expressions of IR ($p < 0.001$), pAkt ($p < 0.001$) and GLUT-1 ($p < 0.001$) was observed in BPS only treated group as compared to control, while BPS plus melatonin treatment showed a significant increase in testicular glucose content ($p < 0.001$), expressions of IR ($p < 0.05$) and pAKT ($p < 0.01$) when compared to BPS only treated group (Fig. 6A-D).

3.10. Effect of BPS and Melatonin treatment on testicular cell proliferation and apoptotic markers

The immunohistochemical and western blot analyses revealed that BPS treatment led to significant ($p < 0.001$) decrease in number of PCNA immunopositive cells and a significant increase in caspase-3 expression ($p < 0.001$) as compared to control. BPS plus melatonin treated group showed a significant ($p < 0.001$) increase in the number of PCNA immunopositive cells and marked reduction in caspase-3 expression ($p < 0.001$) as compared to BPS only treated group (Fig. 7A-G). Furthermore, a significant ($p < 0.001$) increase in number of TUNEL positive cells in BPS only treated group was noted as compared to control while there was significant ($p < 0.001$) decrease in number of TUNEL positive cells in BPS plus melatonin treated group as compared to BPS only treated group (Fig. 8A-M).

4. Discussion

Male subfertility/infertility, builds up psychological and societal pressure that affects not only the sufferers but also their families and communities (Agarwal et al., 2021; Gore et al., 2015). Recent data from the World Health Organization (WHO, 2023) suggests that approximately 17.5% of the adult population worldwide, suffers from infertility.

The expansion of industry over the past few decades has improved our daily life in many ways, but it has also increased occupational and environmental exposure to innumerable chemical compounds, which are known to affect hormonal regulation and are thus regarded as endocrine disrupting chemicals (EDCs). Bisphenol S is an emerging environmental toxicant (Brulport et al., 2021; Gorini et al., 2020; Gyimah et al., 2021; Huang et al., 2019; Kumar et al., 2020; Pal et al., 2023). BPS has gained attention due to its potential to disrupt hormone signalling, however the information about its effects on reproductive organs is limited, especially concerning its molecular insights. Therefore, our aim was to explore the underlying molecular mechanisms behind the adverse effects of BPS on peripheral hormonal balance, testicular function and to examine the protective effects of melatonin.

In accordance with the previous studies (Kumar et al., 2020; Rochester and Bolden, 2015), our data showed a remarkable decrease in body weight and relative testicular weight in mice that were exposed to BPS, indicating potential toxicity on systemic and testicular physiology. Our observations were supported by deteriorated sperm quality, such as reduced sperm count, motility, number of viable sperm, and abnormal sperm morphology. Moreover, the testicular histoarchitectural analyses provided further evidences reinforcing the negative impact of BPS exposure on testicular physiology; as evident by decrease in the height of the germinal epithelium, reduced diameter/ area of seminiferous tubules, germ cells loosening and lumen devoid of spermatozoa. These findings collectively indicate that BPS exposure led to structural and functional alterations in the testes. The anomalies in weight, sperm parameters and testicular histoarchitecture were ameliorated after melatonin supplementation to BPS exposed mice.

Thyroid hormones plays a crucial role in regulating development, differentiation, growth and metabolism in multiple mammalian tissues including testes (Kim and Park, 2019). Earlier, testes was deemed as a thyroid hormone impassive organ but over few decades, increasing evidence has revealed the existence of active thyroid hormone receptors (TRs) and deiodinases in the testes, exemplifying that thyroid hormones play an important role in testicular functions such as spermatogenesis, steroidogenesis, testicular antioxidant defence and survival of germ cells. (Hernandez, 2019; Maran, 2003; Wagner et al., 2009; Wagner et al., 2008). Testosterone plays a critical role in sexual function and spermatogenesis (Barbagallo et al., 2020; Sanderson, 2006). Therefore, alterations in peripheral testosterone, thyroid hormone level, and its receptor expression affect testicular functions and may lead to male infertility. According to earlier studies BPA, BPS, BPF have the potential to disrupt thyroid hormone signalling and can be a molecular target at each stage of the intricate testicular steroidogenesis pathway (Barbagallo et al., 2020; Berto-júnior et al., 2018; Zhang et al., 2018). We noted a significant decrease in serum thyroid hormone (T3/T4) levels, disrupted thyroid histoarchirtecture as evident by significant reduction in follicular epithelial cell height, thinning of follicular epithelial cells, vacuolated follicular epithelium and a significant decline in mRNA expression of steroidogenic enzymes StAR/ CYP11A1 resulting in marked reduction in serum testosterone level, following BPS exposure that impairs spermatogenic cycle. Moreover testicular western blot analyses revealed that BPS administration downregulated expression of thyroid hormone receptor alpha (TR-α) and deiodinase-2 (Dio-2), thereby reduced the bioavailability of active form of thyroid hormone (T3) within the testes. These findings intimate that BPS led to disturbed testicular steroidogenesis and thyroid hormone homeostasis thereby affecting normal testicular functions that may compromise male reproductive health; while BPS plus melatonin treatment showed marked improvement in these parameters ameliorating BPS induced endocrinopathy.

To understand the molecular mechanisms behind testicular toxicity induced by BPS exposure, we extended our study to investigate the regulation of key survival, redox and metabolic modulators. There are burgeoning evidences that BPS can induce oxidative stress by increasing ROS generation in neuronal cells, human red blood cells, rat hepatocytes, mice ovarian tissues (Naderi and Kwong, 2020). Further, BPA and BPS are known to affect mitochondrial electron transport chain augmenting release of ROS, resulting into oxidative stress. SIRT1, NAD-dependent deacetylase plays a pivotal role in regulation of various cellular processes such as survival strategy, redox status, energy metabolism and apoptosis (Merksamer et al., 2013). SIRT1 mediated deacetylation of various transcription factors such as PGC-1α, FOXO-1 and Nrf2 upregulates the expression of antioxidant genes including SOD, Catalase and HO-1 thereby enhancing cell survival. Moreover, melatonin is well-known for its ability to scavenge free radicals directly and indirectly, reduce inflammation, and act as a potent anti-apoptotic molecule (Ferlazzo et al., 2020; Hyun et al., 2023; Kvetnoy et al., 2022). Previous studies have indicated that melatonin is known to modulate SIRT1 expression (Guo et al., 2014; Zheng et al., 2021). Our study also documented noticeable decrease in the mRNA expression as well as protein levels of MT-1 and SIRT1, in BPS treated group which might have reduced mRNA expression of PGC-1α, FOXO-1, Nrf2 and HO-1; while BPS plus melatonin treated group significantly enhanced the expression of these genes, thereby combating oxidative stress condition.

We further investigated additional markers that affect cellular redox balance, such as JAK2/STAT3 signalling. Previous investigation documented the role of JAK2/STAT3 signalling in the cardio protective actions of melatonin (Lan et al., 2019); activation of the JAK2/STAT3 signalling pathway decreases oxidative stress and apoptosis (Yang et al., 2013; Hyun et al., 2023). It is worth noting that mice lacking STAT3 exhibited a shorter lifespan, dysfunctional mitochondria, and excessive reactive oxygen species (ROS) levels, while antioxidant therapy via p-STAT3 expression extended their lifespan (Mantel et al., 2012). Consistent with previous studies, our data showed that BPS exposure significantly reduced the expression of MT-1, p-JAK2 and p-STAT3 which would have also led to testicular oxidative stress as evident by increased lipid peroxidation, elevated levels of nitrite/nitrate, intracellular ROS level in the testes, and reduced activity of antioxidant enzymes i.e., SOD and catalase. However, when melatonin was administered to BPS treated mice we noted marked increase in expression of MT-1, p-JAK2 and p-STAT3 along with antioxidant enzymes, with a concomitant decline in lipid peroxidation, nitrite/nitrate and intracellular ROS level thereby attenuating BPS induced oxidative damage.

Testicular cells being metabolically active tissue requires glucose and its metabolites to sustain the process of spermatogenesis and steroidogenesis (D'Cruz et al., 2012; Rocha et al., 2014). Circulatory glucose is taken up by glucose uptake transporters (GLUTs) specifically GLUT-1/3/8 (Banerjee et al., 2014; Navale and Paranjape, 2016). Insulin plays a pivotal role in cellular uptake of glucose via downstream signalling molecule such as IRS, PI3K, pAKT and GLUTs. Recently, we have reported that BPS exposure has resulted in disturbed glucose homeostasis affecting IR signalling in the ovarian tissue (Pal et al., 2023). In order to understand the effect of BPS on Insulin signalling in testis, we investigated alterations in expression of IR, pAKT and GLUT-1. In present study, we observed that BPS exposure led to a reduction in testicular glucose content, increased serum insulin (hyperinsulinemia) and downregulated expression of IR, pAKT, and GLUT-1; indicating that BPS exposure induces metabolic stress/energy deprivation and testicular insulin resistance state. However, melatonin administration to BPS treated group significantly increased testicular glucose content, normalized serum insulin level and upregulated IR, pAKT, GLUT-1 expression, indicating melatonin administration improved testicular metabolic/energy deprived state.

The regulation of spermatogenesis relies on maintaining a delicate equilibrium between the proliferation and apoptosis of germ cells (Cipolla-Neto et al., 2022; Du et al., 2021). Our study showed that BPS administration resulted in enhanced testicular germ cell apoptosis, as revealed by increase in caspase-3 expression, and number of TUNEL positive cells while a marked decline in testicular proliferating cell nuclear antigen (PCNA) immunopositive cells. However, when melatonin

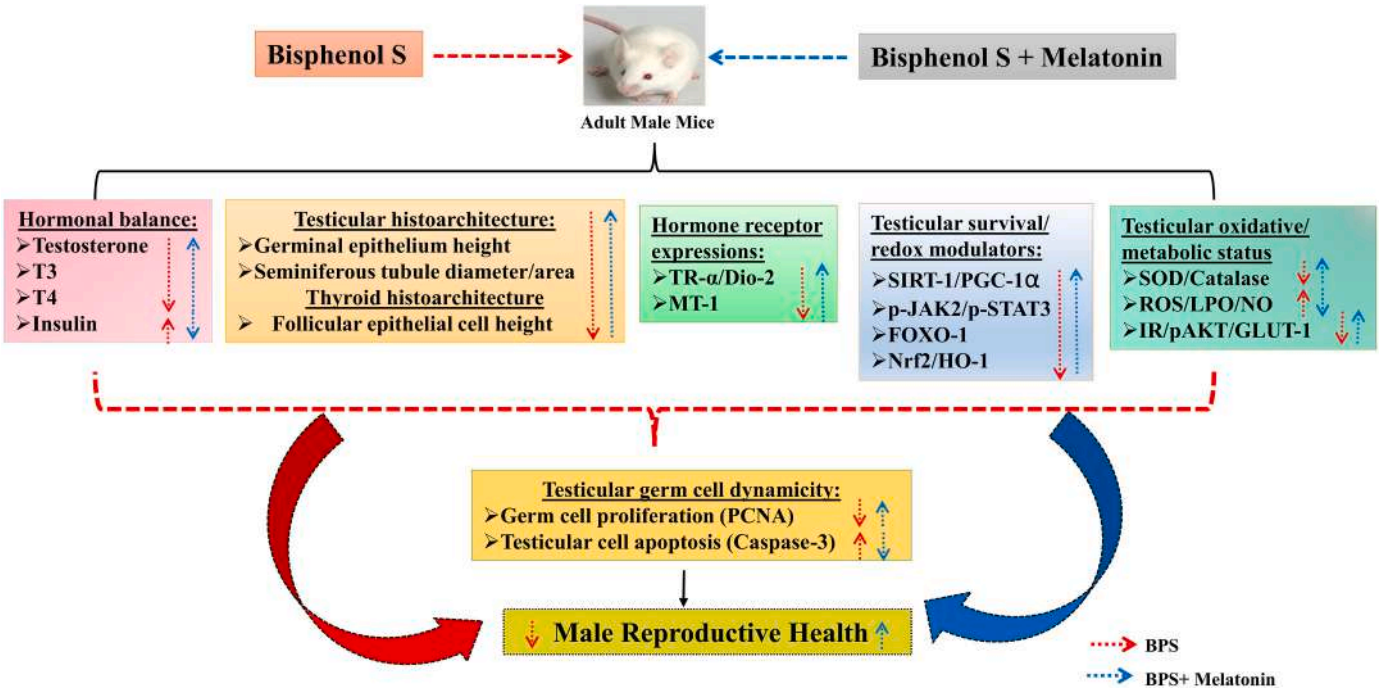

Fig. 9. Schematic diagram representing Bisphenol S induced testicular damages and ameliorative action of melatonin, where red arrow indicates effect of BPS and blue arrows denote effect of melatonin treatment.

was co-administered with BPS, we noticed a remarkable increase in PCNA immunopositive cells, a significant decrease in caspase-3 expression and number of TUNEL positive cells. Thus, it suggests that melatonin treatment was able to promote the proliferation of germ cells, enhancing their survival, and prevented apoptosis.

5. Conclusion

In conclusion, we documented molecular insights of BPS-induced hormonal imbalance and testicular impairment. BPS exposure altered hormonal (testosterone, T3/T4, insulin) balance, sperm parameters, testicular local thyroid hormone homeostasis (TR-α/Dio-2), decreased survival/redox (SIRT1/PGC-1α/FOXO-1, Nrf2/HO-1, p-JAK2/p-STAT3) markers, increased nitro-oxidative load (MDA, nitrite/nitrate), enhanced metabolic abnormalities (IR/pAKT/GLUT-1) and disturbed cellular dynamicity (caspase-3/PCNA), collectively compromising male reproductive potential. In contrast, melatonin treatment alleviated testicular damages caused by BPS via maintaining hormonal homeostasis and counteracting testicular redox/energy deprivation conditions (Fig. 9). Thus our findings reveal that melatonin may be a potent ameliorative candidate against BPS compromised male reproductive health.

CRediT authorship contribution statement

Aishwarya Sahu: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Roles/Writing – original draft, Writing – review & editing. **Rakesh Verma**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Roles/Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank the Council of Science and Technology, Lucknow, UP (CST, UP), Institution of Eminence (IoE-BHU) and CAS in Department of Zoology, BHU for financial assistance to Dr. Rakesh Verma. The authors are thankful to CST-UP for providing financial support to Ms. Aishwarya Sahu as Junior Research Assistant (JRA) fellowship. The authors are thankful for the qPCR facility of the Interdisciplinary School of Life Sciences (ISLS), Banaras Hindu University and Laser Scanning Super Resolution Microscope System (SP8 STED, Leica Microsystems, Germany) facility at Central Discovery Center/ Sophisticated Analytical & Technical Help Institute (CDC/SATHI), Banaras Hindu University.

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