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Pharmacological inhibition of endoplasmic reticulum stress mitigates testicular pathology in a mouse model of simulated microgravity

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ABSTRACT

Background: Simulated microgravity during hindlimb unloaded (HU) induces multi-organ pathologies in mice, including testicular dysfunction. However, a detailed characterization of testicular histology and its driving molecular mechanisms remains elusive. We investigated the potential contribution of elevated endoplasmic reticulum (ER) stress to testicular atrophy in HU mice.

Methods: We divided male c57BL/6j mice into a control group (C) or unloaded mice without treatment (U), or treated with 4PBA (UP), an ER stress inhibitor, for three weeks. At the end of the experiment, mice were euthanized, and testes tissues were dissected for histopathology and mRNA sequencing.

Results: HU was associated with significant testicular atrophy ($p = 0.03$) and several histopathological alterations, including a reduction in the diameter of seminiferous tubules ($p < 0.001$), epithelial thinning ($p < 0.001$), reduced sperm density ($p < 0.001$), and thickening of the epididymal epithelium ($p < 0.001$). mRNA sequencing revealed alterations in several pathways associated with oxidative stress, protein catabolism, and inflammation induction (all $p < 0.05$). Three weeks of treatment with 4PBA prevented testicular atrophy ($p = 0.214$), partly restored testicular microarchitecture, and reversed changes in the transcriptomic profiling of HU mice.

Conclusion: Our novel findings indicate a mechanistic role of elevated ER stress in causing testicular pathology during HU conditions. Based on findings, we report a therapeutic potential of 4PBA in reversing testicular pathology in conditions mimicking prolonged bed rest and spaceflight.

1. Introduction

Prolonged bed rest, spaceflight, and other conditions with reduced mechanical loading of the body induce disruption of several body systems, including male reproductive organs [1,2]. For example, reduced levels of plasma, salivary, and urine testosterone are reported in astronauts following a five-days space mission [3]. Similar effects are reported in following bed rest of two weeks [4] or two to three months [5] in human healthy volunteers. The hormonal inhibition is accompanied by several alterations in microarchitecture and the function of testes in such conditions. Among these, a disruption of seminiferous tubules, reduced sperm count, and lower testosterone production are frequent findings in microgravity conditions [6].

Due to the difficulty of conducting experiments in space, several ground-based models of reduced mechanical loading and microgravity have been developed. The hindlimb unloaded (HU) rodent model exhibits cephalic fluid shift, musculoskeletal unloading, and alterations in several body systems similar to astronauts in spaceflight [7,8]. In addition, the HU rodents also show disruption in testicular microarchitecture, including shrinkage of seminiferous tubules, epithelial thinning, and reduction in spermatocyte count [9]. These alterations are time-dependent and further worsen with the duration of HU [10]. Furthermore, HU also suppresses testosterone production along with a decrease in the expression of androgen receptors and irreversible

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pathological damage to the testes [9,11]. A better understanding of the molecular mechanisms driving testicular pathology in microgravity is required before therapeutic interventions can be tested.

The chronic dysregulation of protein folding by endoplasmic reticulum (ER), a condition called ER stress, has emerged as a critical pathological contributor to various human diseases, including liver, type 2 diabetes, neurodegeneration, and cancer [12,13]. In addition, we have also reported a role for elevated ER stress in HU-induced muscle atrophy [14]. However, how elevated ER stress potentially contributes to testicular pathology in microgravity conditions in HU mice is not clearly known. Nevertheless, several lines of evidence indicate a potential interface between elevated ER stress and testicular pathology. For example, the contribution of prolonged ER stress to infertility in disease conditions has recently been recognized [15,16]. Additionally, the pharmacological inhibition of ER stress with melatonin prevents the apoptosis of spermatogonial stem cells and restores testicular structure and function [17]. Melatonin also exhibits protective effects against testicular injury induced by tunicamycin by suppressing ER stress [18]. Additionally, 4PBA, a pan-ER stress inhibitor, prevents testicular pathology in mice fed on a high-fat diet [19]. These results indicate that the inhibition of ER stress may be an attractive therapeutic target in restoring testicular function under reduced gravity conditions.

To our knowledge, no study has investigated the effects of mitigating ER stress on restoring testicular architecture in HU mice. Moreover, the molecular mechanisms driving testicular disruption in conditions of HU remain poorly understood. Therefore, a detailed dissection of global transcriptomic changes is required to investigate the mechanistic contribution of ER stress to testicular decline in HU. We aimed to investigate the potential contribution of elevated ER stress to testicular pathology in HU mice. We took a mechanistic approach by treating HU mice with 4PBA as an ER stress inhibitor and performing global transcriptomic analysis of testes. We report that three weeks of HU is associated with disruption of testicular microarchitecture, while pharmacological inhibition of ER stress with 4PBA partly restores testicular histology. Using the global transcriptomic profile, we identified key genes and pathways that drive ER stress-induced testicular decline in HU conditions. Altogether, our findings indicate that pharmacological inhibition of ER stress may be an attractive therapy to restore testicular tissues in reduced gravity conditions.

2. Materials and methods

2.1. Animals and HU conditions

We randomly assigned 4-month-old male c57BL/6j mice into ground-based controls (n = 8) and HU mice. HU mice were further divided into two groups, treated with PBS as the vehicle (HU-v; n = 8) or 4PBA (HU-t; 100 mg/kg/d *via* intraperitoneal injections) (n = 8) for three weeks. The dose of 4-PBA was chosen based on previous literature [20,21]. Mice were kept under controlled environmental conditions (20 ± 1 °C, with light/dark periods of 12 h each) with food (standard chow diet for mice) and water provided *ad-libitum,* as described elsewhere by us [22].

2.2. Animals and HU conditions

As described previously, the HU mice were suspended in specially designed cages (AnimalsCareSystems, Colorado, USA) [2,23]. Briefly, the tail was tied to the cage ceiling using a suspension wire, so the hindlimbs were suspended without weight bearing. The sling of the suspension wire ensured free movement throughout the cage on forelimbs. The trunk was at an angle of 30° with the ground. At the end of the experiments, mice were euthanized *via* cervical dislocation, and the testes were immediately harvested and weighed. One testis, along with the epididymis, was fixed in Bouin's and processed for Hematoxylin & Eosin staining, while the other was snap-frozen in liquid nitrogen for further analysis. The experimental protocol was approved by the University ACUC (Animal Care and Use Committee, Reference number: ACUC-19-05-05-01 date June 12, 2019) in agreement with accepted international standards.

2.3. Testicular morphometry

Following a midline abdominal incision, testes were dissected and weighed to assess the presence and potential reversal of damage in the control and experimental groups, respectively. The testicular weights were normalized to body weights to account for variation in body weights. One testis and epididymis were fixed in Bouin's solution and stored in 70% ethanol, then subjected to paraffin embedding, while the other was used for biochemical analysis. The ethanol-fixed tissue was sectioned at 4 μm and further stained with hematoxylin and eosin (H&E) [24]. Morphometric analysis was done as described previously by the PI of this study using a light microscope (Olympus BX 60) [25]. The mean area percentage of collagen fibers content was analyzed using the Image-Pro Plus program (National Institute of Health, Bethesda, MD, USA), while the spermatogenetic activity was evaluated with a light microscope as described in a previous study [25,26].

2.4. Sample collection and library preparation

Total RNA was extracted from the whole testicular tissues of the three groups of mice with Qiagen RNEAsy kit (Qiagen, Valencia, CA, USA). For mRNA sequencing, the total extracted mRNAs were purified with magnetic beads tagged with Ploy-T tail. The first strand and the second were prepared. After synthesizing libraries, they were assessed with Qubit and quantified with real-time PCR. Next, the libraries were analyzed with a bioanalyzer for size detection. Finally, the libraries were pooled, and then sequenced on Illumina platforms, as described previously by us [21].

2.5. Clustering and sequencing

The samples were index-coded before the generation of clusters. The library preparations were sequenced using an Illumina platform to generate paired-end reads, as described elsewhere by us [21].

2.6. Quality control

Raw reads in the FASTQ format were processed by fastp. To get the clean data, reads of the adapter sequence and low quality were removed. Q20 and Q 30 of the clean data were calculated, and all the downstream analysis was performed with good quality data. Using the Spliced Transcripts software (STAR), paired-end clean reads were aligned to the reference genome, as described elsewhere by us [21].

2.7. Quantification

The number of reads that were mapped to each gene was counted using the FeatureCounts. To normalize the reads, Fragments Per Kilobase of transcript sequence per Million (FPKM) fragments mapped were calculated [27]. Any gene with less than 1 FPKM was considered non-expressed in the sample, as described elsewhere by us [21].

2.8. Differential expression analysis

Differential gene expression (DEG) analysis was performed between two conditions/groups (three biological replicates per condition) using the DESeq2 R package. The P values were calculated using Benjamini and Hochberg's method for the False Discovery Rate (FDR). Genes with an adjusted P-value <0.05 were assigned as significantly differentially expressed genes, as described elsewhere by us [21].

2.9. Gene Ontology (GO) and KEGG pathways enrichment analysis

GO enrichment analysis of differentially expressed and uniquely expressed genes between any two conditions was performed by the clusterProfiler R package. GO terms with corrected P-values less than 0.05 were significantly enriched in the differentially expressed and uniquely expressed genes. For the KEGG enrichment analysis, KEGG pathways that were statistically significant adjusted P-value less than 0.05 among the uniquely and differentially expressed genes, as described previously [28].

2.10. Quantitative real-time PCR validation

The qRT-PCR procedure is described in detail elsewhere [29]. Briefly, total RNA was extracted using the RNase kit (Qiagen, Valencia, CA, USA) from 10 mg of frozen testes tissues. cDNA was synthesized using SuperScript II reverse-transcriptase (Life Technologies, Grand Island, NY, USA), and quantitative real-time PCR was performed using SYBR Green PCR Master Mix with primers. The sequence and details of primers are provided in Table 1. We used the delta-delta-ct method to quantify mRNA, as described previously [13].

2.11. Statistical analysis

All numerical values are presented as mean ± SEM. The comparisons among the groups were performed by one-way analysis of variance (ANOVA) and Turkey's multiple comparison tests with a single pooled variance. Data were analyzed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Testes weights

HU was associated with the loss of body weights ($p = 0.003$) (Fig. 1A), absolute testicular weights ($p < 0.0001$) (Fig. 1B), and testicular weights normalized to body weights ($p = 0.03$) (Fig. 1C). 4PBA treatment had no effects on body weight ($p = 0.444$) and testes weight ($p = 0.115$); however, we found no significant reduction in normalized testes weight following 4PBA treatment compared to controls ($p = 0.208$).

3.2. Markers of ER stress

We next investigated the markers of ER stress in HU conditions with or without treatment. HU resulted in an elevated expression of total ($p = 0.021$), spliced ($p = 0.002$), and unspliced XBP-1 ($p = 0.025$) along with ATF4 ($p = 0.001$) (Fig. 1D). However, 4PBA treatment reduced the expressions of spliced XBP-1 ($p = 0.032$) and ATF4 ($p = 0.019$) without affecting total ($p = 0.371$) and unspliced ($p = 0.216$) XP1 (Fig. 1D).

3.3. Testicular histology

We next investigated the testicular histology in the three groups of mice. We observed an orderly progression from spermatogonia to spermatozoa in the seminiferous tubules along with a normal appearance of Sertoli cells, spermatocytes, spermatids, basement membrane, and Leydig cells in ground-based mice (Fig. 2A). Conversely, the testes of HU mice exhibited thinning of luminal diameter ($p < 0.001$) and epithelial height ($p < 0.001$) along with maturation arrests of Sertoli cells and increased vacuolation. In addition, an aggregation of the tails of spermatozoa was found in the seminiferous tubules (Fig. 2B). These morphometric parameters approached the control group in testes of mice treated with 4PBA. These include a partial restoration of normal architecture, demonstrated by an orderly progression from spermatogonia to spermatozoa (Fig. 2C). HU also reduced tubular diameter ($p < 0.001$) (Fig. 2D) and epithelial height ($p < 0.001$) (Fig. 2E), which were incompletely restored with 4PBA treatment (both $p < 0.05$).

We next investigated the effects of 4PBA on the thickness of tunica albuginea in HU mice testes. We found a higher thickness of tunica albuginea following HU ($p < 0.0001$), which was reduced with 4PBA treatment ($p < 0.001$) (Fig. 3A–D). The testes of HU mice also exhibited reduced spermatozoa density ($p < 0.0001$) and the thickness of epididymal epithelia ($p < 0.0001$). However, testes of 4PBA-treated mice had a partial increase in spermatozoa density and thickness of epididymal epithelia (both $p < 0.05$) (Fig. 4A–E). However, the 4PBA-treated HU mice still demonstrated reduced sperm density ($p = 0.009$) and thickness of epididymal epithelia ($p = 0.003$) than the ground-based controls.

3.4. Bulk transcriptomic analysis of the testis

The bulk transcriptomic analysis was performed on total RNAs extracted from the testis in the three conditions. Fig. 5 indicates the differential expressed genes among the three conditions. Surprisingly, there were only 41 differentially expressed genes between the ground control and unloading testis. The differential expression was calculated as the mean of three samples; the low number of the differentially expressed genes might indicate the inconsistency in the expressions of several differentially expressed genes in the testis during the unloading. The PCA and the hierarchical clustering analysis (Fig. 6A and B) exhibit that the mRNA sequencing of the unloading samples are scattered comparatively widely in contrast to the gene expressions of UP and C samples. However, there were 41 differentially expressed genes and several uniquely expressed genes that were consistently differentially or uniquely expressed between unloading and control samples.

In contrast, 2414 genes were differentially regulated between ground control and treated mice, indicating the potent effect of the ER stress inhibitor on the expression of several genes. The supplementary file 1 contains all the differentially expressed genes among three conditions. The main GO terms and KEGG pathways enriched in the differentially expressed genes are listed in Fig. 6. The main GO terms enriched in the upregulated genes in the unloading testis compared with control were DNA and RNA helicase-related terms. A total of 1046 out of 2414 differentially expressed genes were upregulated between treated and

Table 1
The sequences of primers used in real-time PCR, annealing temperatures, length, and amplicon sizes.

Sr. No.	Gene bank Number	Primers	Tm	Length	Amplicon Size	References
1	>NM_001289726.2	GAPDH (F): CATCACTGCCACCCAGAAGACTG	63.28	23	153 bp	[30]
		GAPDH (R): ATGCCAGTGAGCTTCCCGTTCAG	65.15	23		
2	>NM_009716.3	ATF4 (F): GGGTTCTGTCTTCCACTCCA	58.94	20	96 bp	[31]
		ATF4 (R): AAGCAGCAGAGTCAGGCTTTC	60.88	21		
3	>NM_013842.3	Xbp1 (F): CAGCACTCAGACTATGTGCA	57.62	20	76 bp	[31]
		Xbp1 (R): GTCCATGGGAAGATGTTCTGG	58.35	21		
4	>NM_001271730.1	S-Xbp1 (F): CTGAGTCCGAATCAGGTGCAG	60.74	21	324 bp	[31]
		S-Xbp1 (R): GTCCATGGGAAGATGTTCTGG	58.35	21		

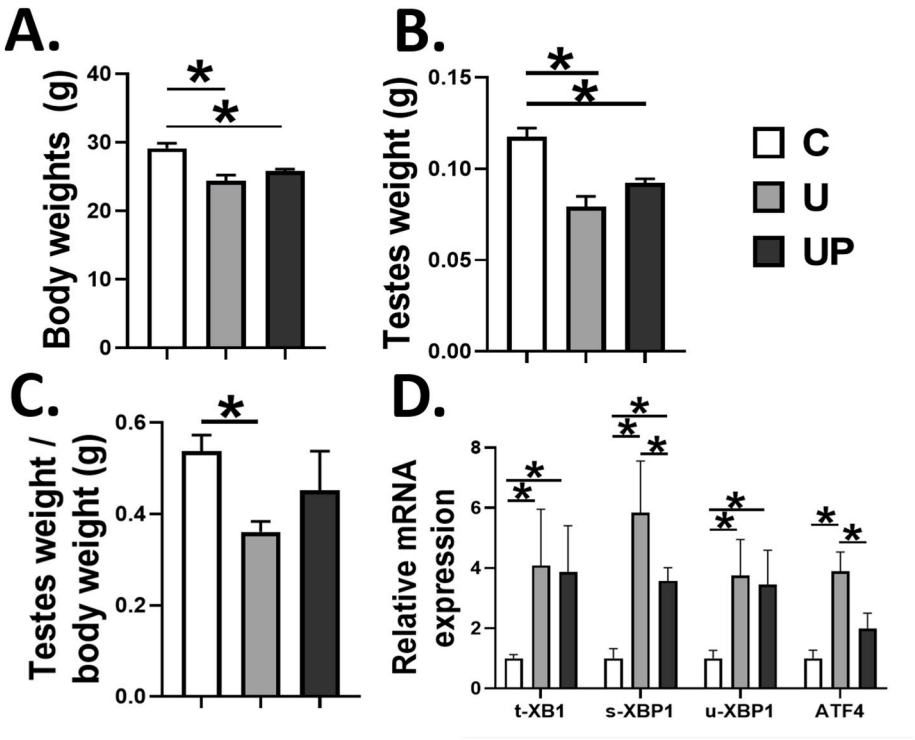


Fig. 1. Body weights (A), absolute (B), and relative (C) testes weights and expressions of markers of ER stress in ground-based controls (C) and unloaded mice without treatment (U) or treated with 4PBA (UP) for three weeks. Data is represented as Mean ± SEM (n = 10–12/group). One-way analysis of variance, *p < 0.05.

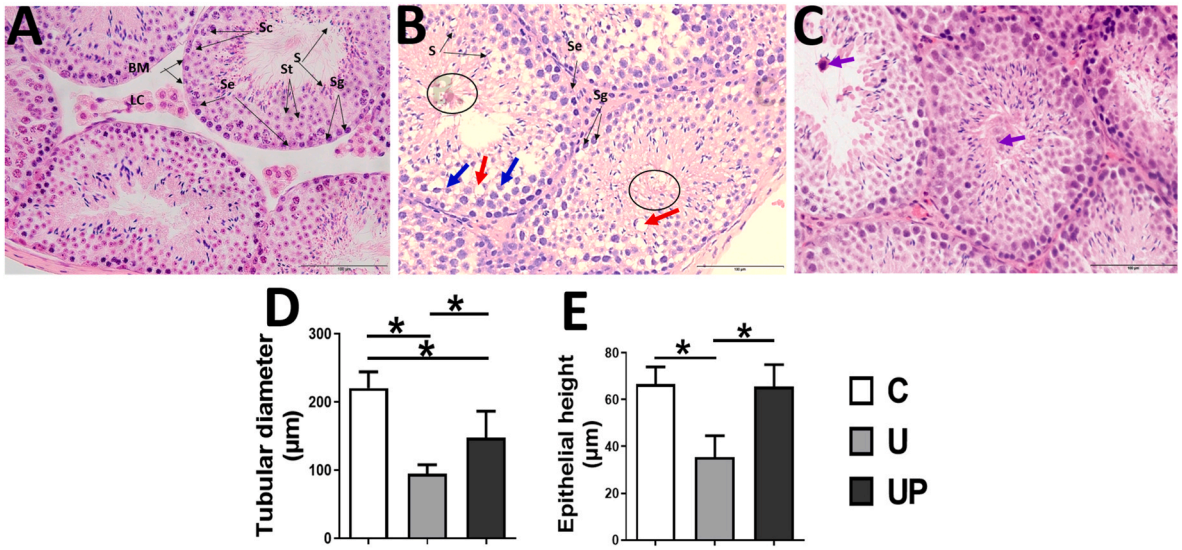


Fig. 2. H&E images of testes from ground-based controls (A) and unloaded mice without treatment (B) or treated with 4PBA (C) for three weeks. A show the Normal cellular architecture with orderly progression from spermatogonia (Sg) to spermatozoa (S) in the seminiferous tubules. Normal Sertoli cells (Se), Spermatocytes (Sc), Spermatids (St), Basement membrane (BM), and Leydig cells (LC). B shows closely packed nuclear chromatin with maturation arrest (blue arrow) or vacuolation (red arrow) of Sc. The black circle indicates the absence of the lumen with aggregation of the tail of spermatozoa. C shows pyknotic bodies implying sloughed cells into the lumen (purple arrows). All images were taken at 40× magnification. The tubular diameter (D) and epithelial heights (E) are represented as Mean ± SEM (n = 10–12/group) (Scale bar = 100 μm). One-way analysis of variance, *p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ground controls, and these upregulated genes enriched several GO terms and KEGG pathways. The main GO terms enriched included microtubules, Ras protein signal transduction, the stem cell population maintenance, Golgi vesicle transport, regulation of Rho protein signal transduction, and Golgi vesicle transport. The 4PBA enhanced these GO and other Terms during the partial recovery of the testis (Fig. 7). The KEGG pathway analysis revealed that aldosterone synthesis and secretion and the cAMP signaling pathway are activated due to the 4PBA treatment. The down-regulated 1368 genes between treated and ground-based controls enriched several GO terms and KEGG pathways. The main GO terms enriched in the down-regulated genes were structural constituents of the ribosome, ribosome, mitochondrion organization, and respiratory chain complex. The KEGG pathways, such as ribosome and oxidative phosphorylation pathways, were also enriched in the

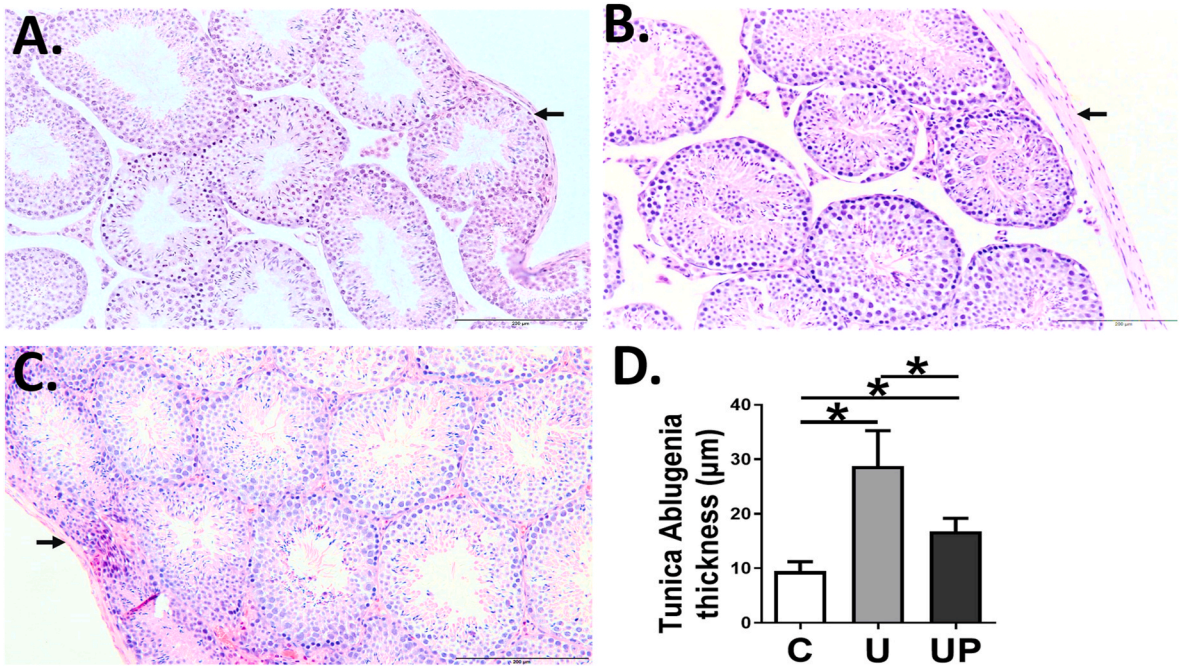


Fig. 3. H&E images of testes from ground-based controls (A) and unloaded mice without treatment (B) or treated with 4PBA (C) for three weeks. The black arrow indicates the changes in the thickness of tunica albuginea. The thickness of tunica albuginea from the three groups of mice (D) is Mean ± SEM. All images were taken at 40× magnification (n = 10–12/group). (Scale bar = 100 μm), One-way analysis of variance, *p < 0.05.

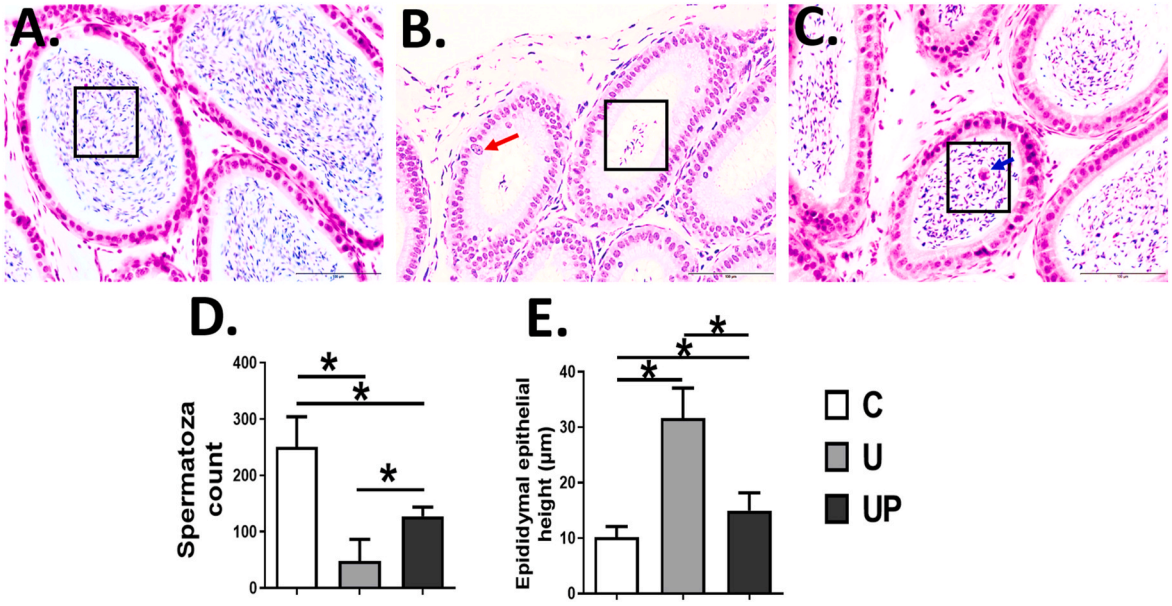


Fig. 4. H&E images of epididymis from ground-based controls (A) and unloaded mice without treatment (B) or treated with 4PBA (C) for three weeks. The boxes show sperm densities. The red arrow indicates a multinucleated giant cell. The spermatozoa count (D) and the epithelial heights of the epididymis (E) are represented as Mean ± SEM. All images were taken at 40× magnification (n = 10–12/group). (Scale bar = 100 μm), One-way analysis of variance, *p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

genes that were downregulated in the control (Fig. 7). Supplementary files 2 and 3 list the enriched GO terms and KEGG pathways, respectively, among the three conditions.

Since the expressions of many genes varied in the unloading samples, we also analyzed uniquely expressed genes among the three conditions. Any gene with an average of less than 1 FPKM was considered switched off. Fig. 8 describes the Venn diagram of the uniquely expressed genes in the three comparisons. Supplementary file 4 contains all the uniquely expressed genes among the three conditions. GO terms and KEGG pathways enrichment analysis was also performed on these uniquely expressed genes in the three conditions. Unloading switched on 887 genes while switching off 443 genes in the testis compared to the ground condition. The primary GO terms enriched in the 887 unique genes in the unloading were positive regulation of cell proliferation, regulation of the metabolic process, and response to stress. Since the unloading switch on many genes, some other GO terms that may not be relevant to testis atrophy were also enriched (Fig. 9). The KEGG pathway enrichment analysis shows that the 887 genes were associated with the activation of

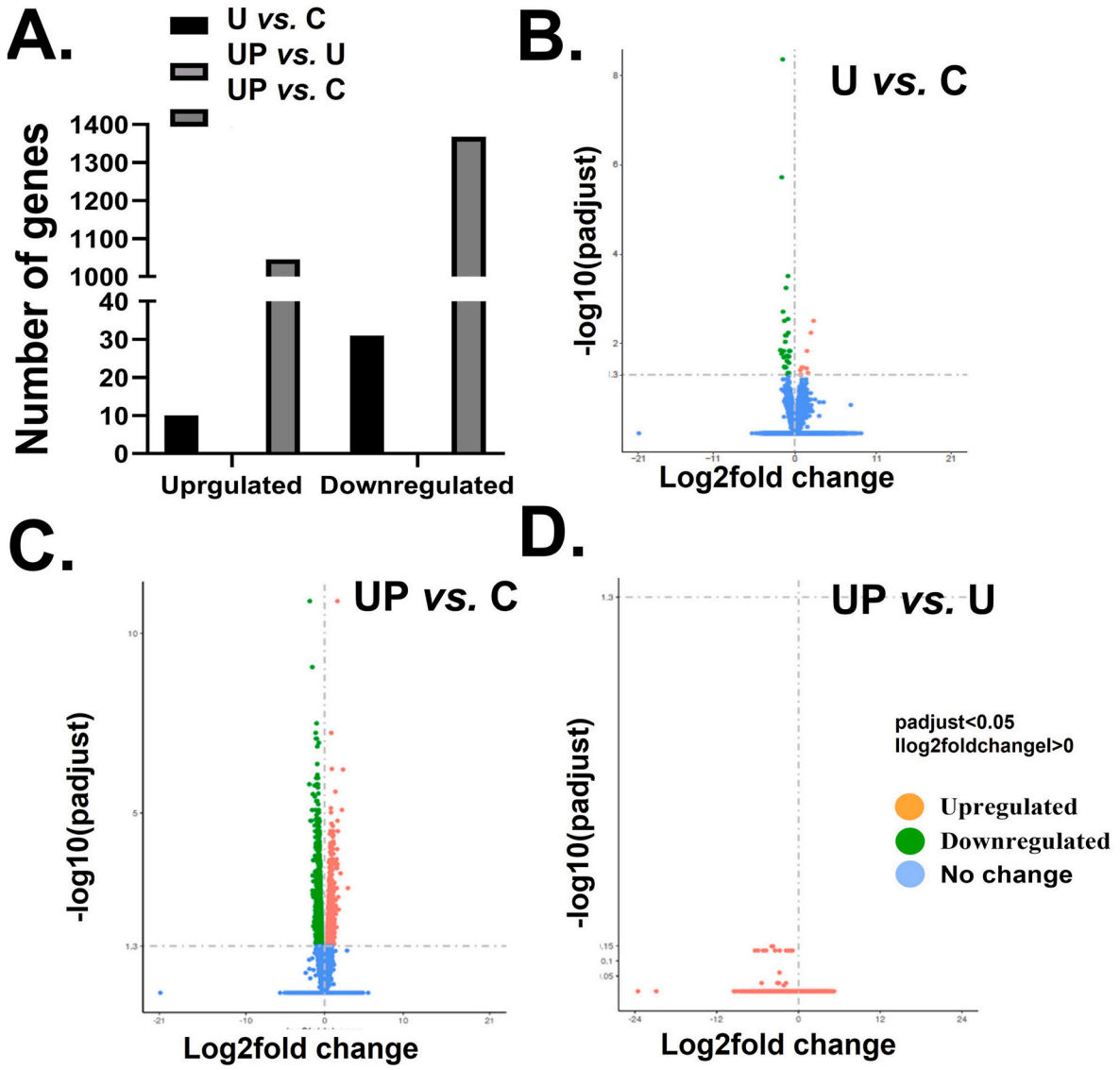


Fig. 5. The total number of differentially expressed genes from ground-based controls and unloaded mice without treatment or treated with 4PBA (A). Volcano plots of the differentially expressed genes between unloaded and controls (B), unloaded mice treated with 4PBA and controls (C), and the two subgroups of unloaded mice (D).

the metabolic, Rap 1, and Calcium signaling pathways. The testis atrophy might lead to the activation of these pathways; however, further research is warranted to elucidate the roles of these pathways in testis pathology. Switching off 443 genes in the unloading testis did not enrich any GO term or KEGG signaling pathway.

A total of 604 genes were uniquely expressed in unloading compared to the treated condition. The main GO terms enriched in these uniquely expressed genes were associated with extracellular regions, response to stress, regulation of proteolysis, defense response, and oxidation-reduction process (Fig. 9). The main KEGG pathways enriched in these 604 genes were associated with metabolic pathways, phagosomes, PPAR signaling pathways, antigen processing, and presentation. Conversely, 4PBA treatment partially recovered the testis by regulating these processes and pathways. The 4PBA also switched on 463 genes in the treated testis compared to the testis in the unloading condition. However, these 463 uniquely expressed genes did not enrich any GO term.

Histological studies indicate that 4PBA partially recovered the testis pathology in the unloading testis. To unravel the molecular mechanism of this recovery, the uniquely expressed genes of treated and control groups were also analyzed; 779 unique genes in the treated while 476 unique genes in the control group were reported. The KEGG pathway enrichment of the 779 genes shows the involvement of metabolic and other signaling pathways, calcium, cGMP-PKG, and oxytocin (Fig. 9). The activation of these pathways further indicates the potent effects of the 4PBA on various signaling pathways, which lead to partial recovery of the testis. The 476 uniquely expressed genes in the control compared to the treated sample group did not enrich any GO term. The supplementary files 5 and 6 list all the significantly enriched GO terms and KEGG pathways in the uniquely expressed genes among the three conditions.

4. Discussion

We report a significant disruption of normal testicular microarchitecture under HU conditions, including reduced tubular diameter, epithelial thinning, reduction in spermatozoa density, and thickening of tunica albuginea. These effects may be attributed to reduced gravitational loading and cephalic fluid shift, resulting in hypoxia of testes. At molecular level, elevated ER stress appears to be a critical driver of these effects. This was evident by the partial reversal of most of these changes

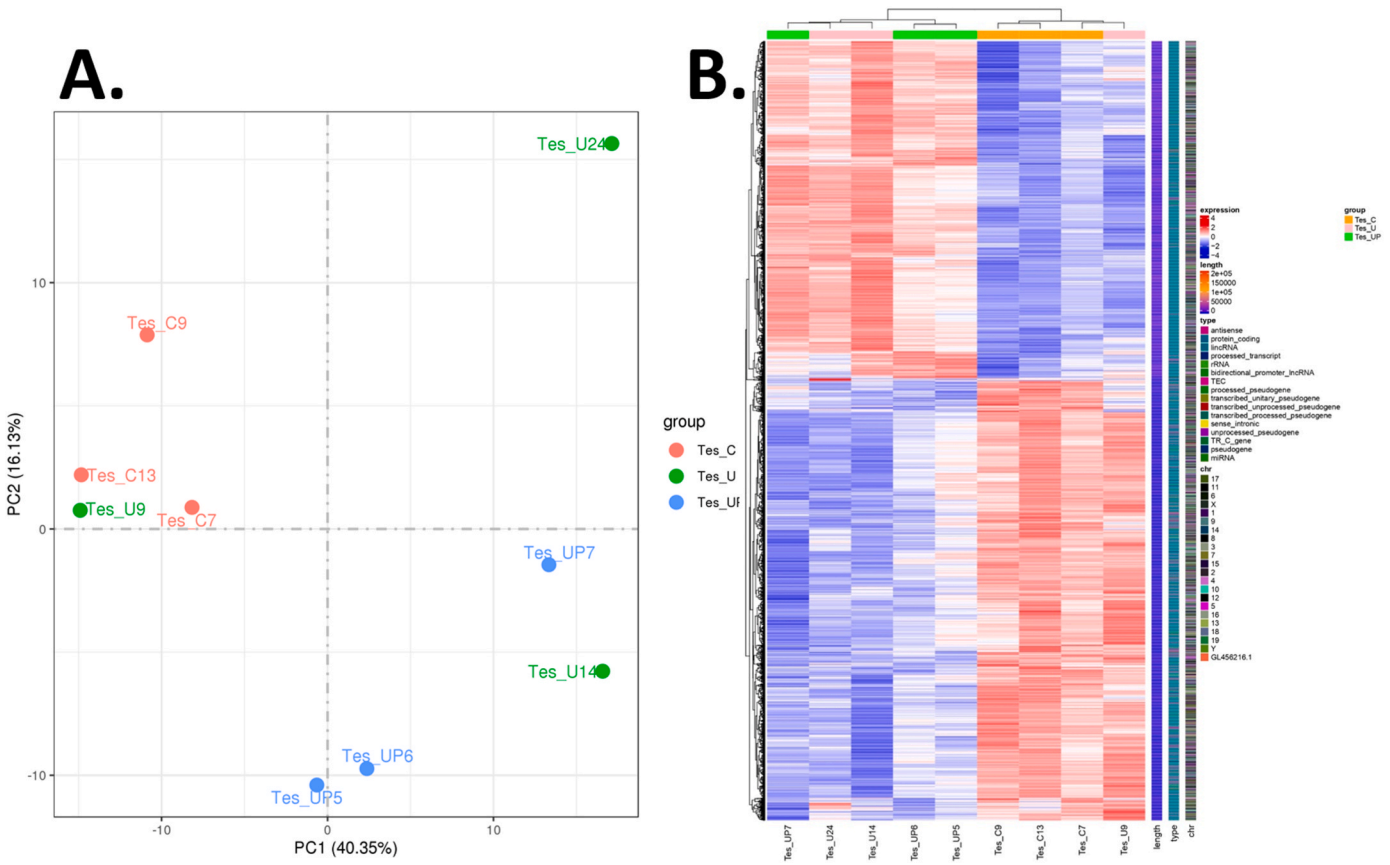


Fig. 6. A: PCA Plots of the mRNA seq of the samples according to the gene expressions (A) and the Hierarchical clustering of gene expressions in the ground-based controls and unloaded mice treated with vehicle or 4PBA.

with 4PBA treatment. Additionally, transcriptome analysis revealed several candidate pathways and genes involved in testicular pathology and its reversal following HU and 4PBA treatment, respectively.

We first confirmed the potency of 4PBA with a reduction in the ER stress markers in the treatment group. We also observed the prevention of testicular atrophy in the treated group, indicating the therapeutic efficacy of 4PBA. An attenuated ER stress also prevented several micro abnormalities in the testicular architecture. Specifically, restoration of tubular diameter, epithelial thickness, and spermatozoa count in the testes of HU mice was observed. No direct evidence of the beneficial effects of 4PBA on testes has been reported. However, our findings are consistent with the protective effects of 4PBA on mouse Sertoli cells in an *in-vitro* model [32]. Specifically, 4PBA prevented ER stress and cellular apoptosis in these cells, which is consistent with our findings of the protective effects of 4PBA on mouse testes.

We did not dissect the individual downstream pathways of ER stress, including UPR arms in HU testes. However, we found an elevation of s-XBP1, which was reversed with 4PBA treatment. This can lead to the activation of IRE1 sensors and the pro-apoptotic pathways [33]. Interestingly, ER stress can induce apoptosis through a second pathway involving PERK/ATF4/CHOP [34]. This pathway is the first to be activated in response to a noxious stimulus and is not observed in chronic pathologies lasting for months [33]. Considering the three weeks of HU, we expected an upregulation of ATF4 in HU testes. Accordingly, we found a higher expression of ATF4 following HU, which was reversed with 4PBA treatment. We also hypothesized the activation and/or upregulation of apoptosis in HU conditions. The unique expression of pro-apoptotic caspase 7 in HU testes confirmed this hypothesis. This finding can also partly account for cellular and histological detriment observed in the HU testes. On the other hand, we did not find a unique expression of caspase 7 following 4PBA treatment, confirming the association between the abundance of s-XBP1 and activated apoptosis in HU testes.

We established a causal association between ER stress and testicular pathology using 4PBA, which partly reversed the histopathological changes in the mouse testes. These findings complement the scarce data about testicular microarchitecture in the HU rodents [9]. Specifically, the HU rats exhibit several features of testicular pathology, including scarcity of spermatozoa, formation of syncytial structures, loosening of the basal lamina, and loss of germinal cells [9].

We performed a transcriptomic analysis of the testes to investigate the potential mechanistic links between ER stress and testicular pathology. The analysis of differentially expressed pathways revealed several candidates accounting for ER stress-induced testicular pathology in the HU mice. Specifically, several pathways associated with mitochondrial homeostasis were upregulated in the 4PBA-treated HU mice. Conversely, similar changes were not found with HU in the untreated group. The contribution of disrupted mitochondrial homeostasis to testicular pathology has recently been recognized [35]. Specifically, mitochondrial dysregulation led to reduced bioenergetics and elevated oxidative stress, which is implicated in testicular pathology. Thus, mitochondrial dysregulation can potentially account for the interface between elevated ER stress and testicular pathology in HU conditions. It is also well known that mitochondrial disruption can increase cellular oxidative stress, which is a critical trigger of testicular atrophy and dysfunction in multiple disease conditions [36]. Additionally, increased oxidative stress and testicular atrophy have previously been reported in HU rodents [9,37]. Thus, countering oxidative stress can improve testicular function and sperm density [36]. Our transcriptomic analysis revealed a unique expression of genes associated with reducing oxidant capacity. Thus, the restoration of mitochondrial function and resultant cellular bioenergetics and antioxidant capacity can provide a

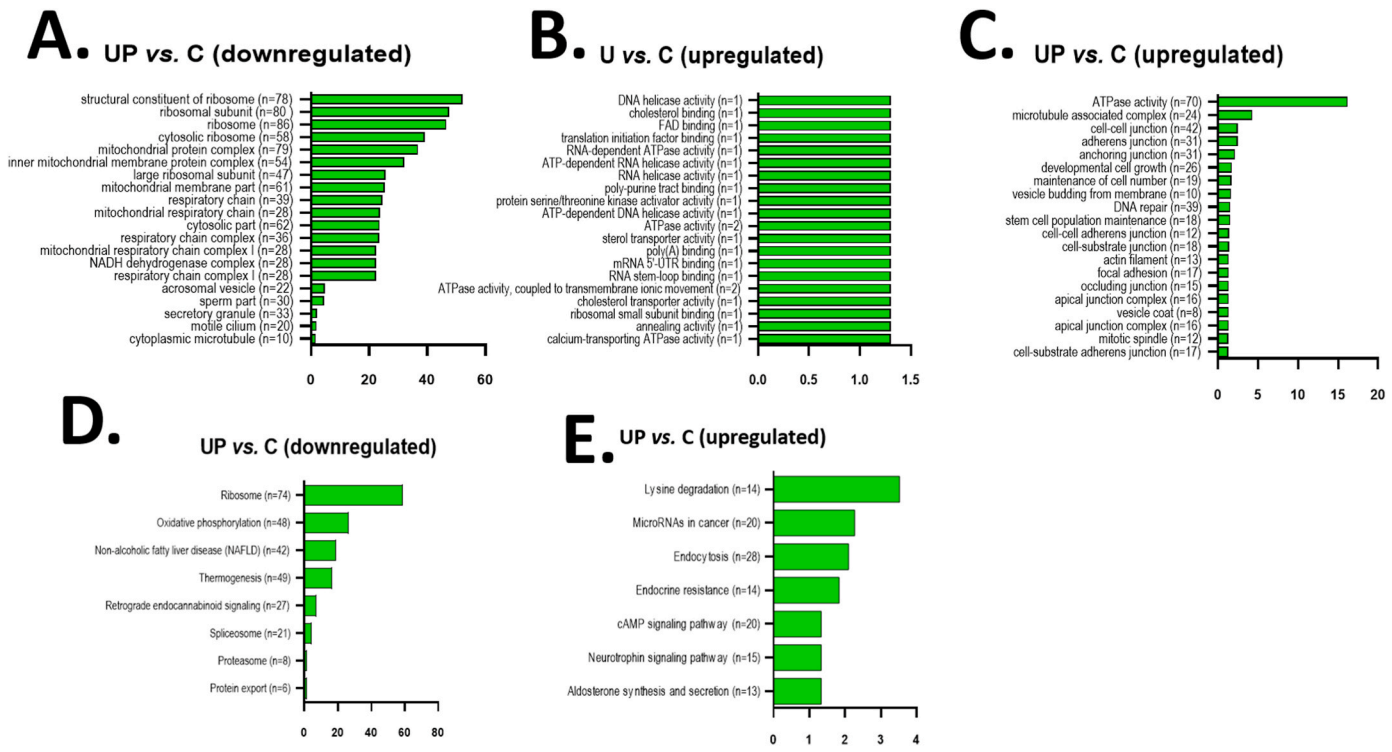


Fig. 7. GO terms and KEGG pathways enrichment analysis of the differentially expressed genes. Top significant and relevant GO terms enriched in the downregulated genes of unloaded mice treated with 4PBA and ground-based controls (A). Top significant and relevant GO terms enriched in the upregulated genes of unloaded and ground-based control mice (B). Top significant and relevant GO terms enriched in the upregulated genes of unloaded mice treated with 4PBA and ground-based controls (C). Top significant and relevant KEGG pathways enriched in the downregulated genes of unloaded mice treated with 4PBA and ground-based controls (D) and top significant and relevant KEGG pathways enriched in the upregulated genes of the unloaded mice treated with 4PBA and ground-based control mice.

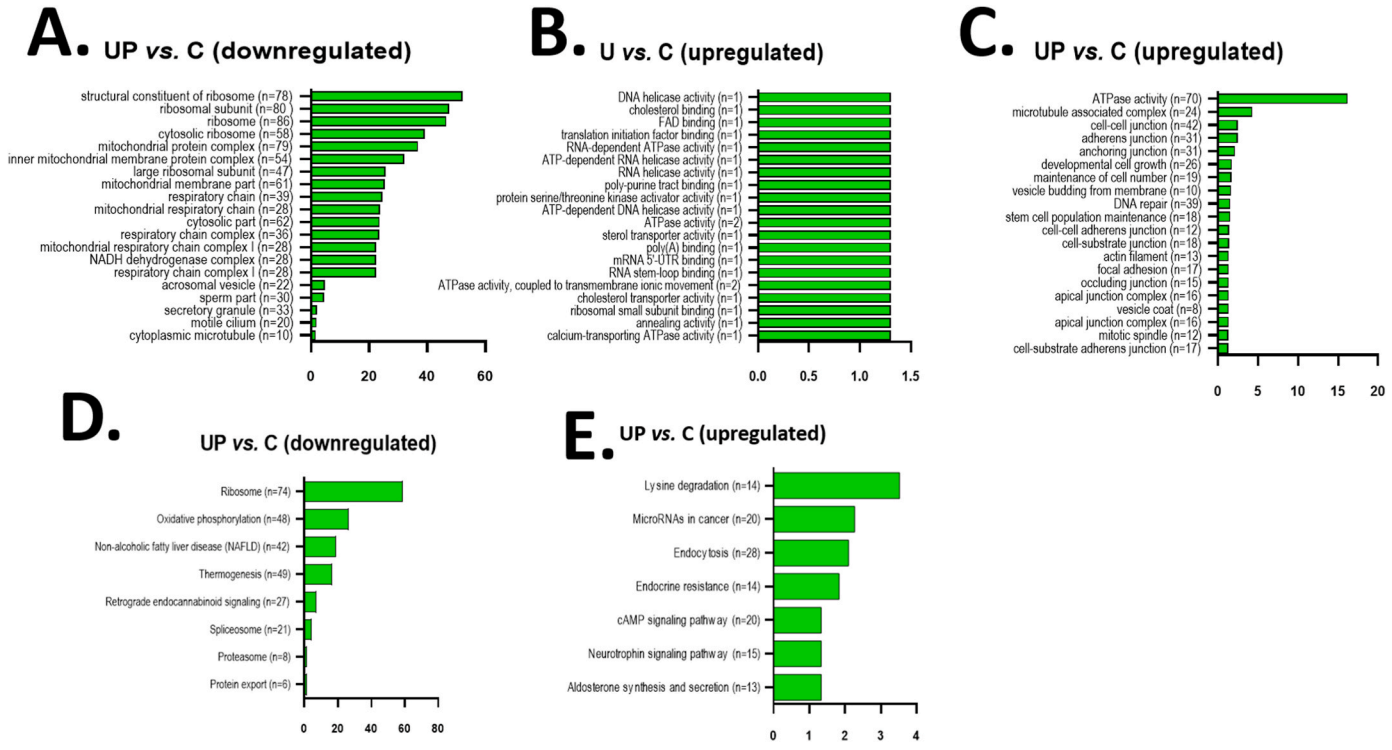
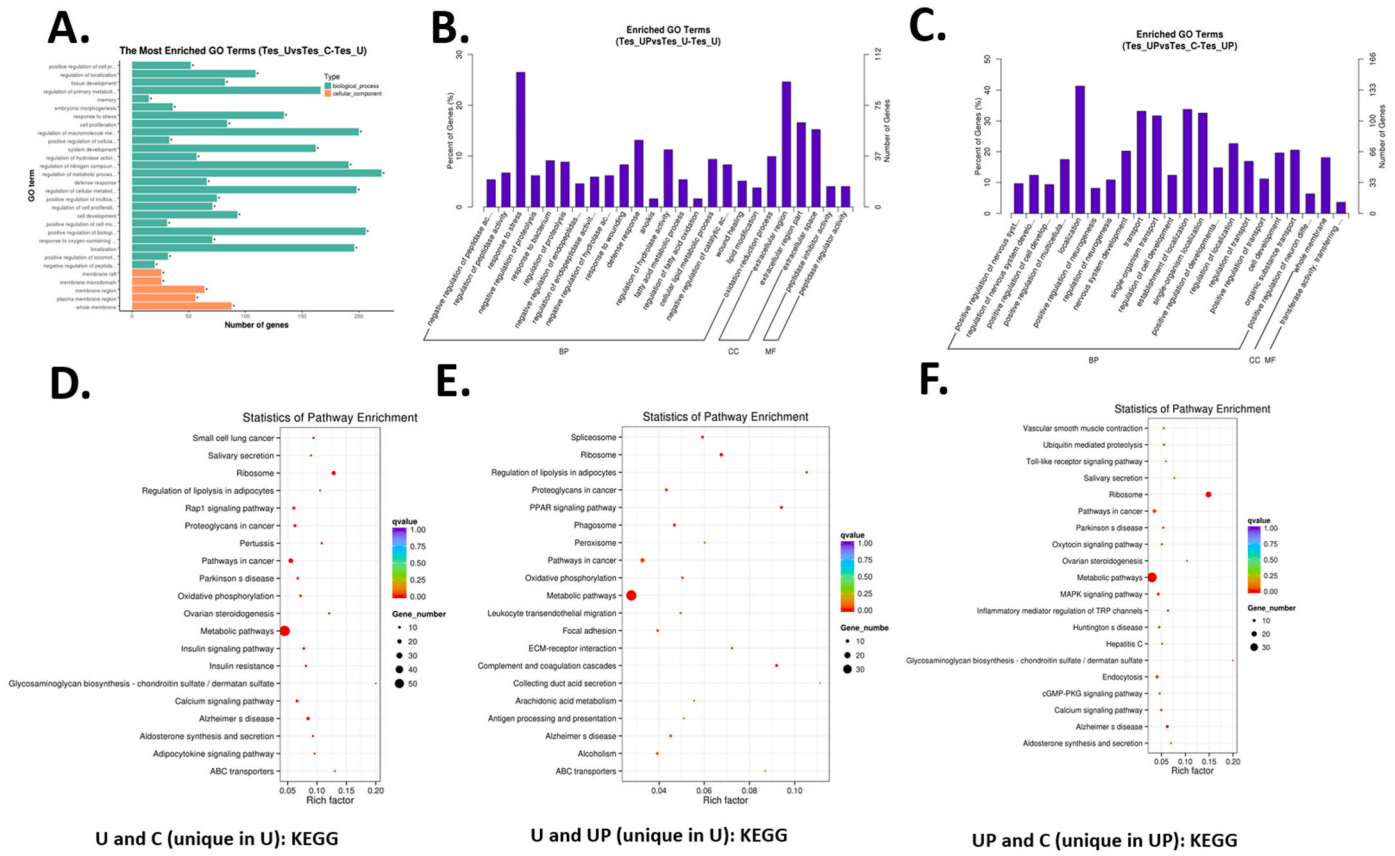


Fig. 8. Venn diagrams of the uniquely expressed genes comparing the unloaded vs. ground-based controls (A), unloaded mice treated with 4PBA vs. ground-based controls (B), and the two subgroups of unloaded mice (C).



U and C (unique in U): KEGG

U and UP (unique in U): KEGG

UP and C (unique in UP): KEGG

Fig. 9. GO terms and KEGG pathways enrichment analysis of the uniquely expressed genes. Top significant and relevant GO terms enriched in the uniquely expressed genes of unloaded vs. controls mice (A), top significant and relevant GO terms enriched in the uniquely expressed genes of the two subgroups of unloaded mice (B), top significant and relevant GO terms enriched in the uniquely expressed genes of unloaded mice treated with 4PBA vs. ground-based control mice (C), top significant and relevant KEGG pathways enriched in the uniquely expressed genes of unloaded mice compared with control mice (D), top significant and relevant KEGG pathways enriched in the uniquely expressed genes of the two subgroups of unloaded mice (E), and top significant and relevant KEGG pathways enriched in the uniquely expressed genes of unloaded mice treated with 4PBA compared with control mice (F).

mechanistic explanation of the effects of 4PBA in HU testes. We also found a unique expression of genes mediating the regulation of metabolic pathways, response to stress, and negative regulation of peptidase activity in the HU mice. A few classic pathways, such as the PI3K-Akt signaling pathway, MAP-Kinase (MAPK) signaling pathway, and cAMP signaling pathway, were upregulated in the unloading testis.

We found a unique expression of several genes associated with cellular catabolism and inflammation in the untreated HU mice compared to the 4PBA-treated group. These are consistent with the report showing that 4PBA treatment suppresses apoptosis in Sertoli cells [32]. In addition, 4PBA is also shown to reduce inflammation [38], which can partly account for its protective effects in the HU testes. An increase in inflammation is a critical downstream consequence of elevated ER stress and may contribute to histopathological findings in the HU testes. In support of this, several studies report an association of elevated inflammation with testicular pathologies [33,39,40]. The unique expression of Toll-like receptors (TLRs) signaling pathway in the HU *vs.* than unloaded mice is consistent with this finding. TLRs signaling is involved in maintaining immune homeostasis and inflammatory response in the testes [41].

Moreover, we found a unique expression of the MAPK signaling pathway in the HU testes compared to the unloaded group. MAPK pathway performs several functions in testes, including germ cell differentiation and progression, regulation of blood-testes-barrier, and Sertoli cell regulation [42]. The activation, differentiation, and progression of spermatozoa by MAPK are consistent with a higher sperm density in the histology sections of the UP testes. These findings unravel a unique role for 4PBA in restoring sperm density by activating the MAPK pathway. In addition to its regulatory function on germ cells, MAPK regulates cellular metabolic adaptation to various stimuli [43]. Interestingly, 4PBA induced a unique expression of genes associated with metabolic pathways in the HU mice. While we did not investigate individual metabolic pathways in detail, our findings indicate a role for metabolic alterations in preventing testicular dysfunction in HU conditions.

The major strength of this study is the use of a standardized mouse model to investigate testicular pathology and ER stress. We used 4PBA as an ER stress inhibitor, a well-recognized drug for suppressing ER stress in multiple organs. The global transcriptomic analysis we conducted provides a generalized mechanistic insight into ER stress and testicular pathology. The experiments were conducted in a standardized setting with minimal variations among the groups. However, this study has certain limitations. Due to intraperitoneal administration of 4PBA, it may have multiple off-target effects on other organs. 4PBA can directly regulate cellular epigenetics independent of its effects on ER stress [44]. However, we did not investigate epigenetic modifications in this study. In addition, the protective effects of 4PBA may extend to other organs, which may have positive effects on testes due to well-established multi-organ crosstalk in physiological systems. However, our study was focused on the direct effects of 4PBA on testicular tissues during HU conditions. In addition, it is possible that molecular mechanisms other than elevated ER stress may partly be responsible for HU induced-testicular pathology. For example, testicular dysfunction can partly be mitigated without a reduction in ER stress in an experimental rat model of varicocele [45]. Thus, ER stress may not be the only driver of testicular disruption in HU conditions. However, we did not

investigate other potential mechanisms of testicular pathology in HU mice. We also did not rigorously dissect the downstream consequences of ER stress, including individual UPR arms. However, the aim of this study was to relate pan-ER stress with testicular pathology without investigating individual arms of ER stress and UPR. We did not measure the expressions of additional markers of ER stress and UPR. However, the splicing of XBP-1 is considered as a standard molecular marker of elevated ER stress in multiple tissues [31], including skeletal muscles [21]. Spliced XBP-1 has a relatively short half-life of 22 min [46], which may have affected our measurements. However, this short half-life is adequate for transcriptional activation of target genes, so we are confident about the activation of ER stress and its downstream consequences in HU mice.

Altogether, our novel findings elaborate on the mechanistic role of elevated ER stress in testicular pathology in HU conditions. We report several candidate pathways driving testicular atrophy and dysfunction during HU conditions. We also propose 4PBA administration as a potential therapeutic intervention to offset testicular atrophy and dysfunction in conditions mimicking HU, such as prolonged bed rest and spaceflight. Future studies are required to further dissect the mechanistic effects of ER stress in testicular disruption.

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the University ACUC (Animal Care and Use Committee, Reference number: ACUC-19-05-05-01 date June 12, 2019) in agreement with accepted international standards.

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analyzed during this study have been deposited in the GEO database NCBI (https://www.ncbi.nlm.nih.gov/geo/), with the accession number: GSE205126 (Temporary reviewer's token: uhepkaukjrmtjqp).

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Authors' contribution

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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.

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Not Applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.actaastro.2023.01.011.

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