Supplementary Information for

Plant-Derived Exosome-Like Nanovesicles Alleviate Testicular Injury by Suppressing p21-mediated Cell Cycle Arrest in Sertoli Cells

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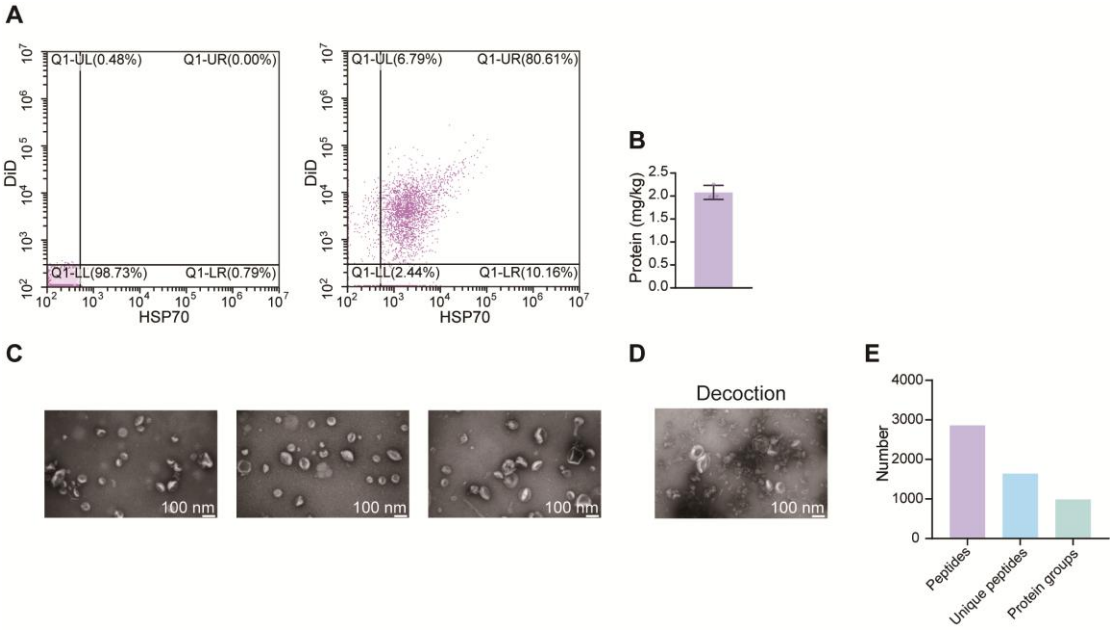
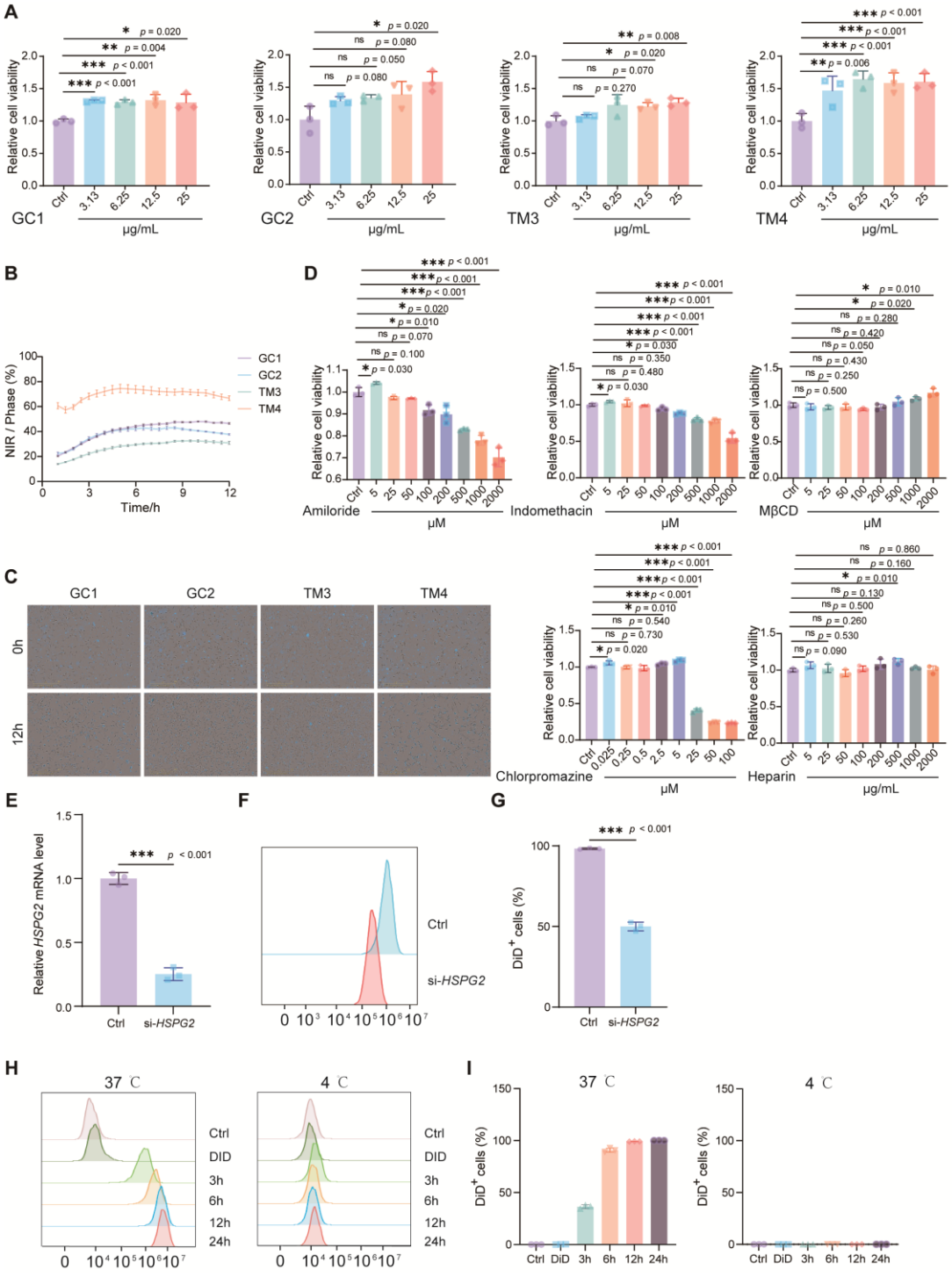
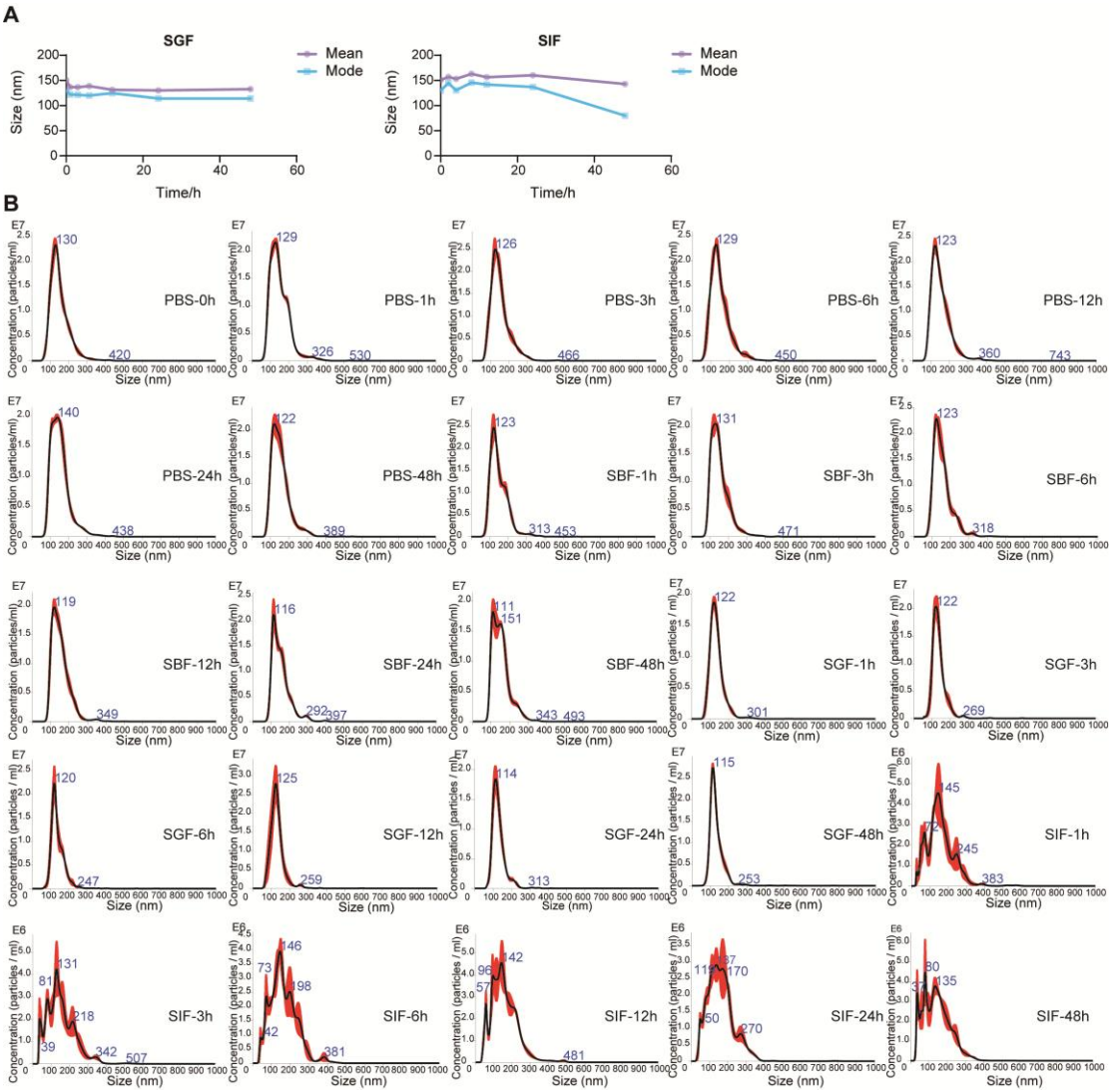


Supplementary Figure 1. Characterization of CDELNs. (**A**) CDELNs were stained with DiD and HSP70, followed by detection using nano-flow cytometry. (**B**) Quantification of CDELNs yield from *Cistanche deserticola* by protein concentration determined with the BCA assays ($n = 3$). (**C**) TEM images of CDELNs derived from different sampling batches. (**D**) TEM images of CDELNs derived from water decoction. (**E**) Statistical analysis of the number of identified peptides and proteins in the proteomic profiling of CDELNs. Data are presented as the means ± SDs.

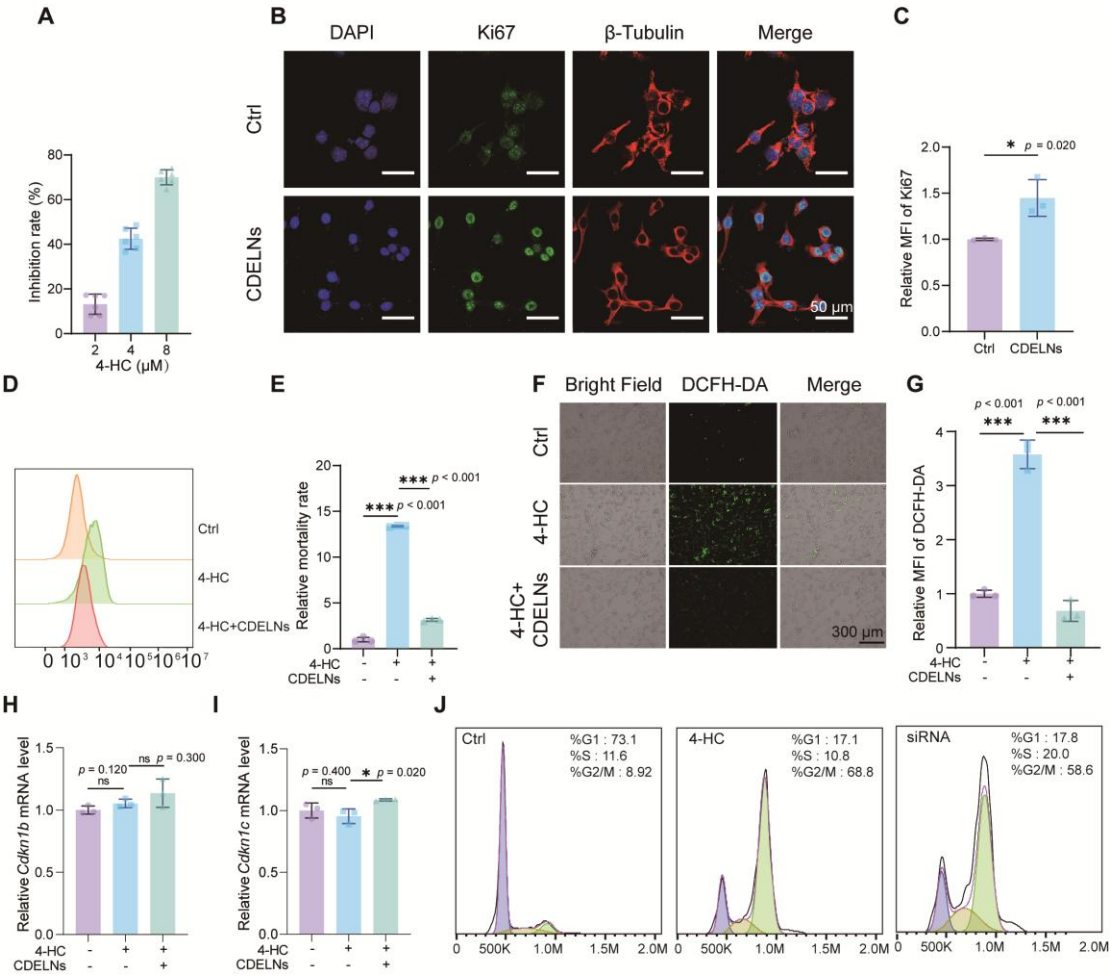


Supplementary Figure 2. In vitro cytotoxicity and cellular uptake of CDELNs. (**A**) Cytotoxicity of CDELNs on four testicular cell lines (GC1, GC2, TM3, TM4) by MTT assay (n = 3). (**B**) Cellular uptake of DiD-CDELNs in four testicular cell lines by long time-lapse imaging. (**C**) Bright-field images of four testicular cell lines treated with DiD-CDELNs. (**D**) Cytotoxicity of various endocytic inhibitors (amiloride, indomethacin, MβCD, chlorpromazine, heparin) on TM4 cells by MTT assay (n = 3). (**E**) qRT-PCR analysis of *HSPG2* expression in TM4 cells. (**F**) Flow cytometric analysis of control TM4 cells and HSPG2-knockdown TM4 cells treated with DiD-labeled CDELNs. (**G**) Quantitative analysis of DiD-

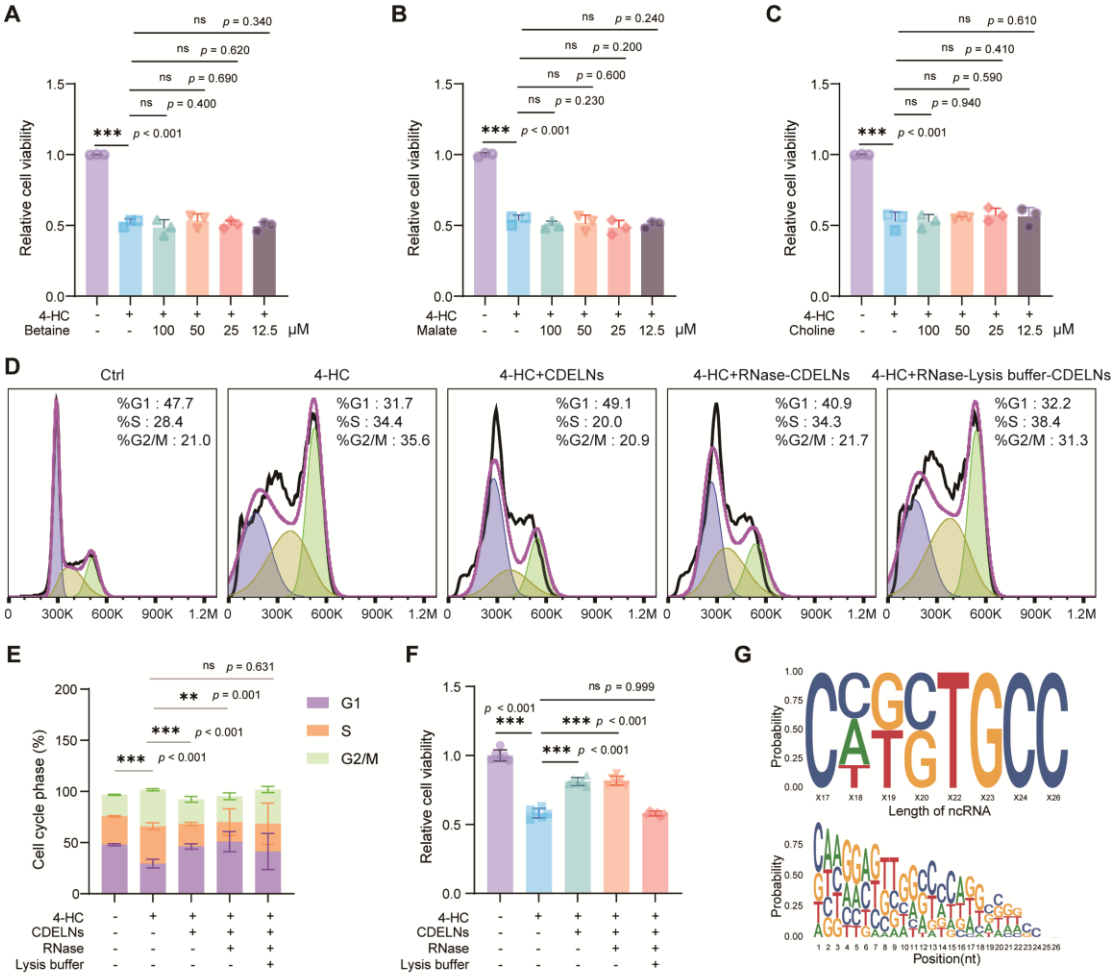
CDELNs^{+} cells in (**F**) (n = 3). (**H**) Flow cytometric analysis of TM4 cells treated with DiD-labeled CDELNs for different durations at 37°C and 4°C. (**I**) Quantitative analysis of DiD-CDELNs^{+} cells in (**H**) (n = 3). Data are presented as the means ± SDs. Differences between groups were assessed by one-way ANOVA followed by Dunnett's post-hoc test or unpaired Student's t-test as appropriate, with $p < 0.05$ defined as statistically significant.



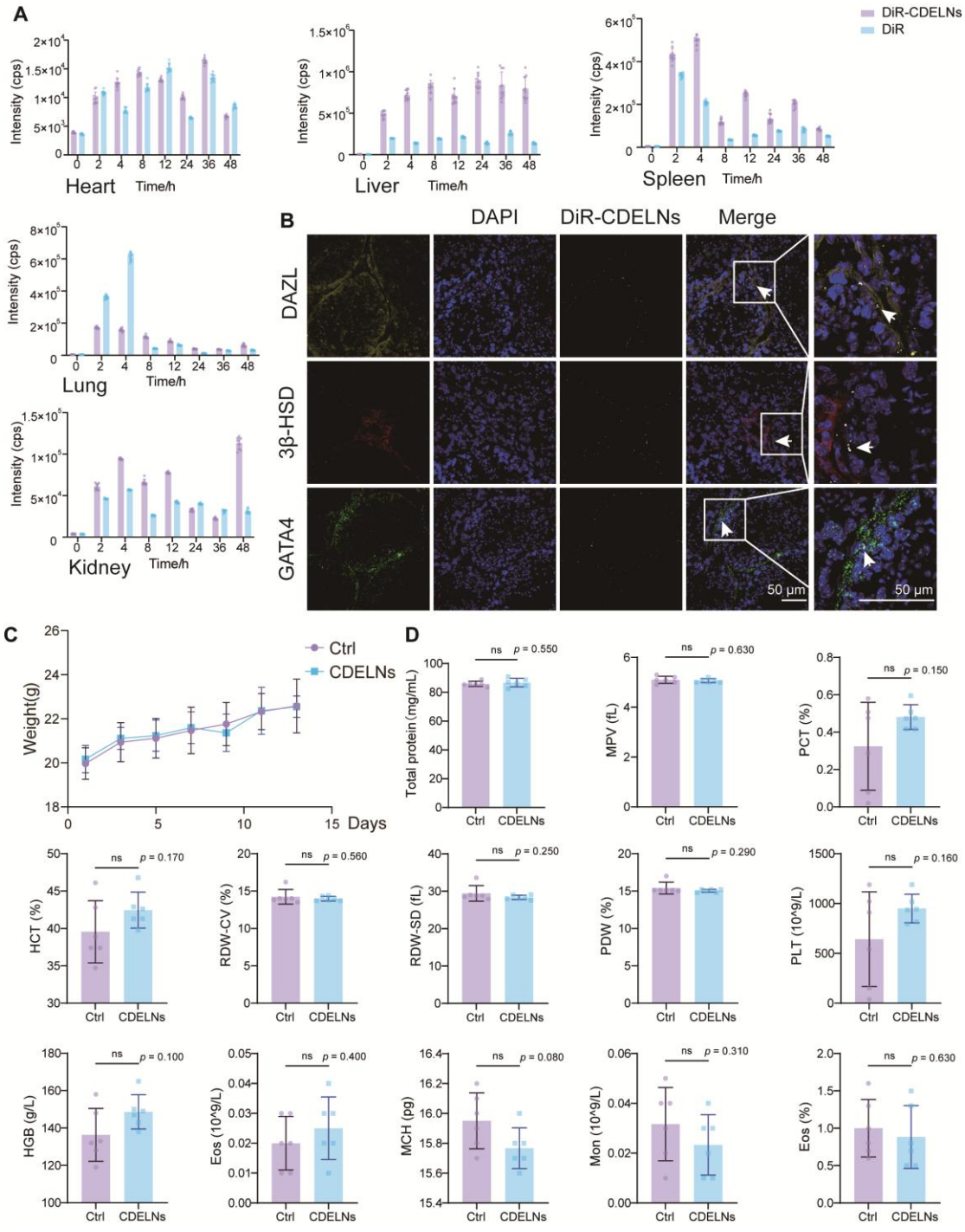
Supplementary Figure 3. Stability of CDELNs under different conditions. (**A**) Changes in the mean particle sizes and mode sizes of CDELNs after incubation in Simulated gastric fluid (SGF) or Simulated intestinal fluid (SIF) at 37 °C for 1 h, 3 h, 6 h, 12 h, 24 h and 48 h. (**B**) Size distribution of CDELNs incubated in PBS, simulated body fluid (SBF), SGF and SIF for different durations (1, 3, 6, 12, 24, and 48 h) using nanoparticle tracking analysis.



Supplementary Figure 4. CDELNs alleviated 4-HC-induced TM4 cell injury in vitro. (**A**) Inhibition rates of TM4 cells treated with different concentrations of 4-HC. (**B**) Representative fluorescence images of Ki67 in TM4 cells following CDELNs intervention. (**C**) Quantitative analysis of mean fluorescence intensity of Ki67 in (**B**) (n = 3). (**D**) CDELNs ameliorated 4-HC induced cell death in TM4 cells by flow cytometric analysis using Zombie Aqua viability dye. (**E**) Quantitative analysis of mean fluorescence intensity of Zombie Aqua dye in (**D**) (n = 3). (**F**) Representative fluorescence images of reactive oxygen species (ROS) in 4-HC-treated TM4 cells following CDELNs intervention. (**G**) Quantitative analysis of mean fluorescence intensity of ROS in (**F**) (n = 3). (**H, I**) qRT-PCR analysis of *Cdkn1b* (**H**) and *Cdkn1c* (**I**) expression in 4-HC-treated TM4 cells following CDELNs intervention. (**J**) Cell cycle analysis of 4-HC-treated TM4 cells after transfection with *Cdkn1a* siRNAs by flow cytometric analysis. Data are presented as the means ± SDs. Differences between groups were assessed by one-way ANOVA followed by Dunnett's post-hoc test or unpaired Student's t-test as appropriate, with $p < 0.05$ defined as statistically significant.

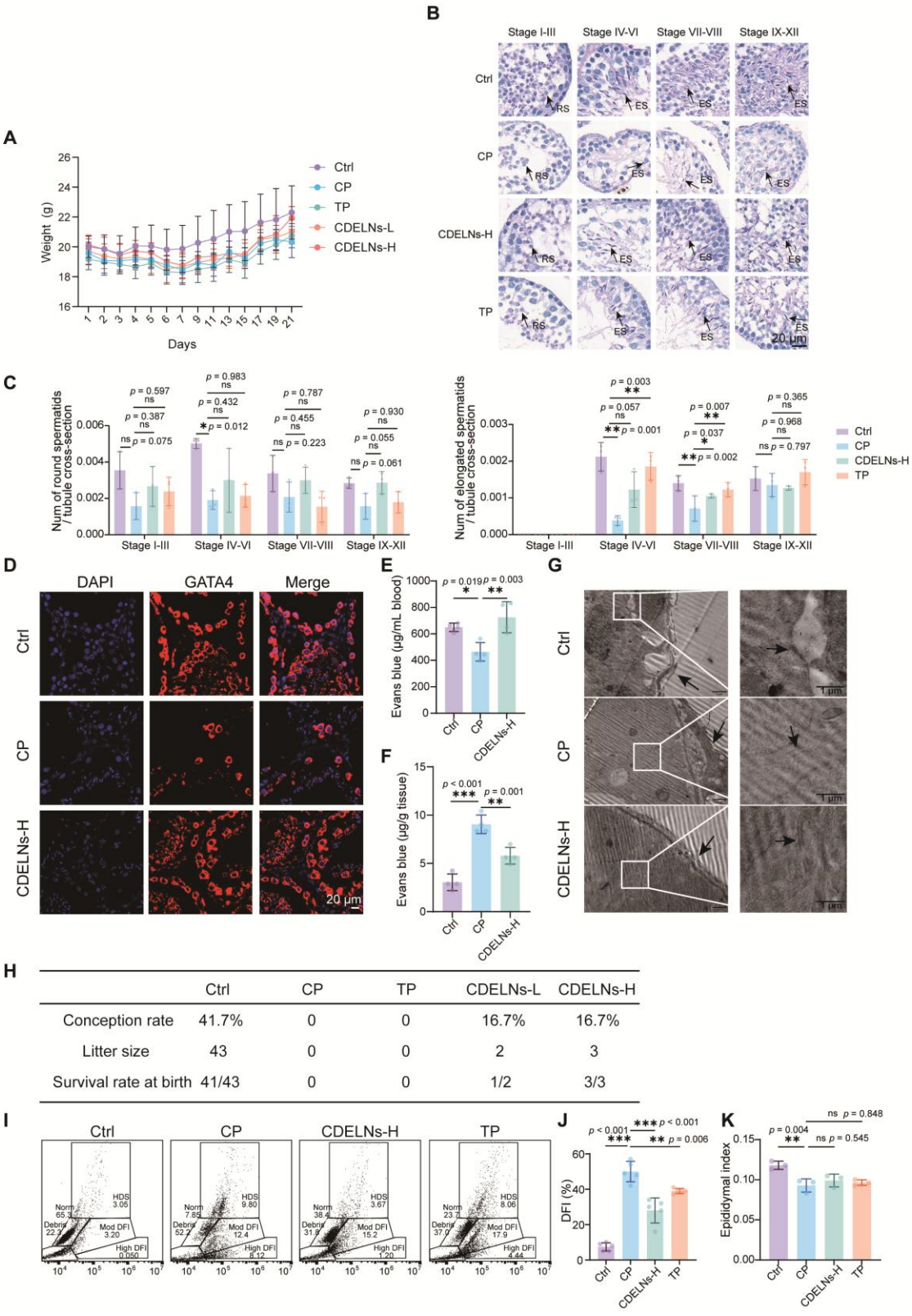


Supplementary Figure 5. Identification of bioactive components in CDELNs. (**A**–**C**) Cell viability of 4-HC-treated TM4 cells following betaine (**A**), malate (**B**), and choline (**C**) interventions by MTT assay (n = 3). (**D**) CDELNs or RNase-treated intact CDELNs relieved 4-HC induced cell cycle arrest in TM4 cells by flow cytometric analysis. (**E**) Quantitative analysis of cell cycle phase in (**D**) (n = 3). (**F**) CDELNs or RNase-treated intact CDELNs increased cell viability against 4-HC induced injury in TM4 cells. (**G**) First nucleotide bias of known miRNAs (17–26 nt) in CDELNs (Top). miRNA nucleotide bias at each position (Bottom). Data are presented as the means ± SDs. Differences between groups were assessed by one-way ANOVA followed by Dunnett's post-hoc test as appropriate, with $p < 0.05$ defined as statistically significant.



Supplementary Figure 6. Biodistribution and biosafety assessment of CDELNs in vivo. (**A**) Fluorescence intensity of DiR or DiR-CDELNs in mouse heart, liver, spleen, lung, kidney. Data are presented as the means ± SEMs. *n* = 10 technical replicates. (**B**) Representative immunostaining images of DAZL (a spermatogenic cell marker), 3β-HSD (a Leydig cell marker), and GATA4 (a Sertoli cell marker) in testicular sections from CP-treated mice under the interventions of CDELNs. (**C**) Body weight of mice measured over 14 days following intraperitoneal administration of CDELNs (*n* = 6). (**D**) Blood routine test after 14 days of intraperitoneal injection of CDELNs, including Total protein, Mean Platelet Volume (MPV),

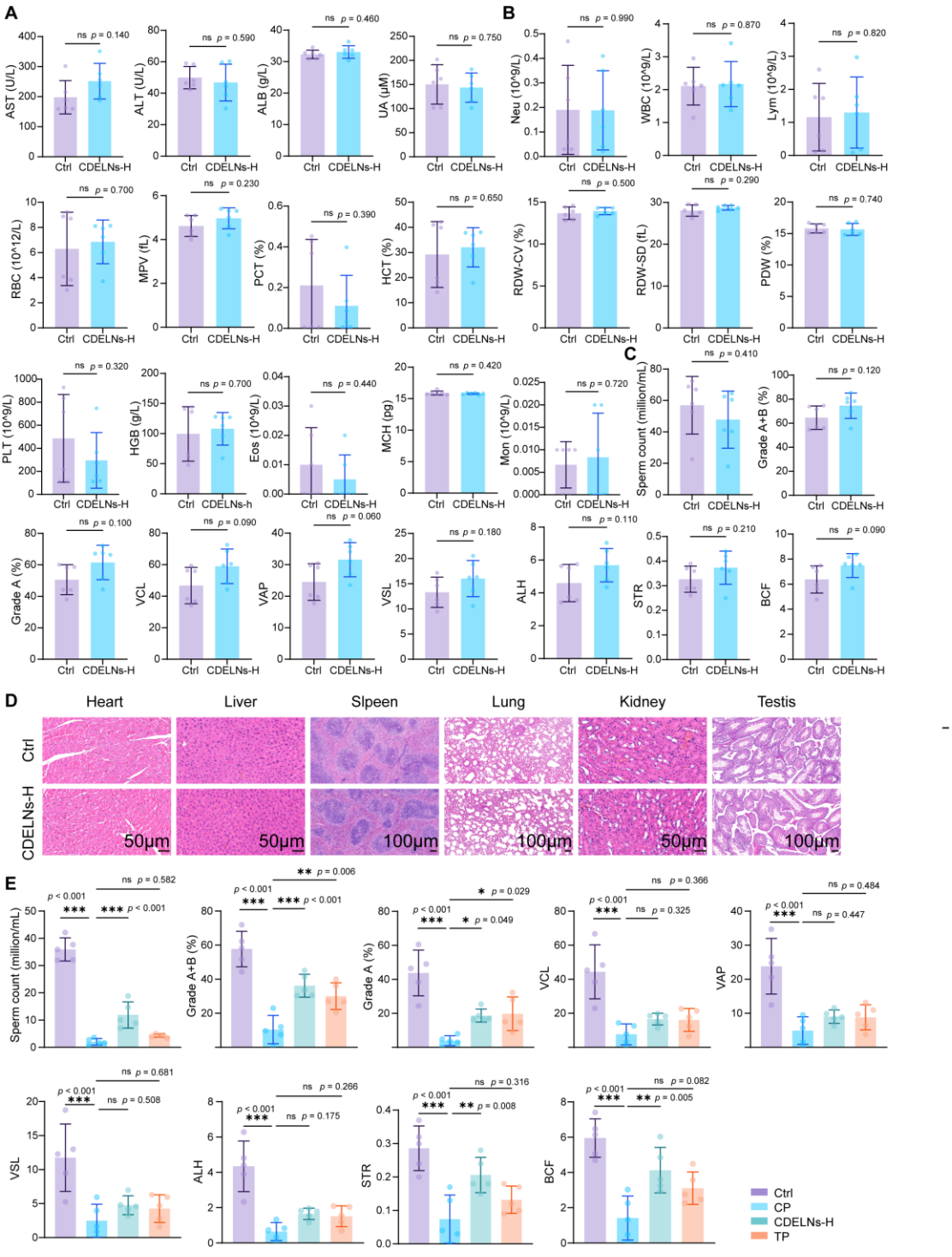
Plateletcrit (PCT), Hematocrit (HCT), Red Cell Distribution Width-Coefficient of Variation (RDW-CV), Red Cell Distribution Width-Standard Deviation (RDW-SD), Platelet Distribution Width (PDW), Platelet (PLT), Hemoglobin (HGB), Eosinophil (Eos), Mean Corpuscular Hemoglobin (MCH), and Monocyte (Mon) (n = 6). Data are presented as the means ± SDs. Differences between groups were assessed by one-way ANOVA followed by Dunnett‘s post-hoc test or unpaired Student’s t-test as appropriate, with $p < 0.05$ defined as statistically significant.



	Ctrl	CP	TP	CDELNs-L	CDELNs-H
Conception rate	41.7%	0	0	16.7%	16.7%
Litter size	43	0	0	2	3
Survival rate at birth	41/43	0	0	1/2	3/3

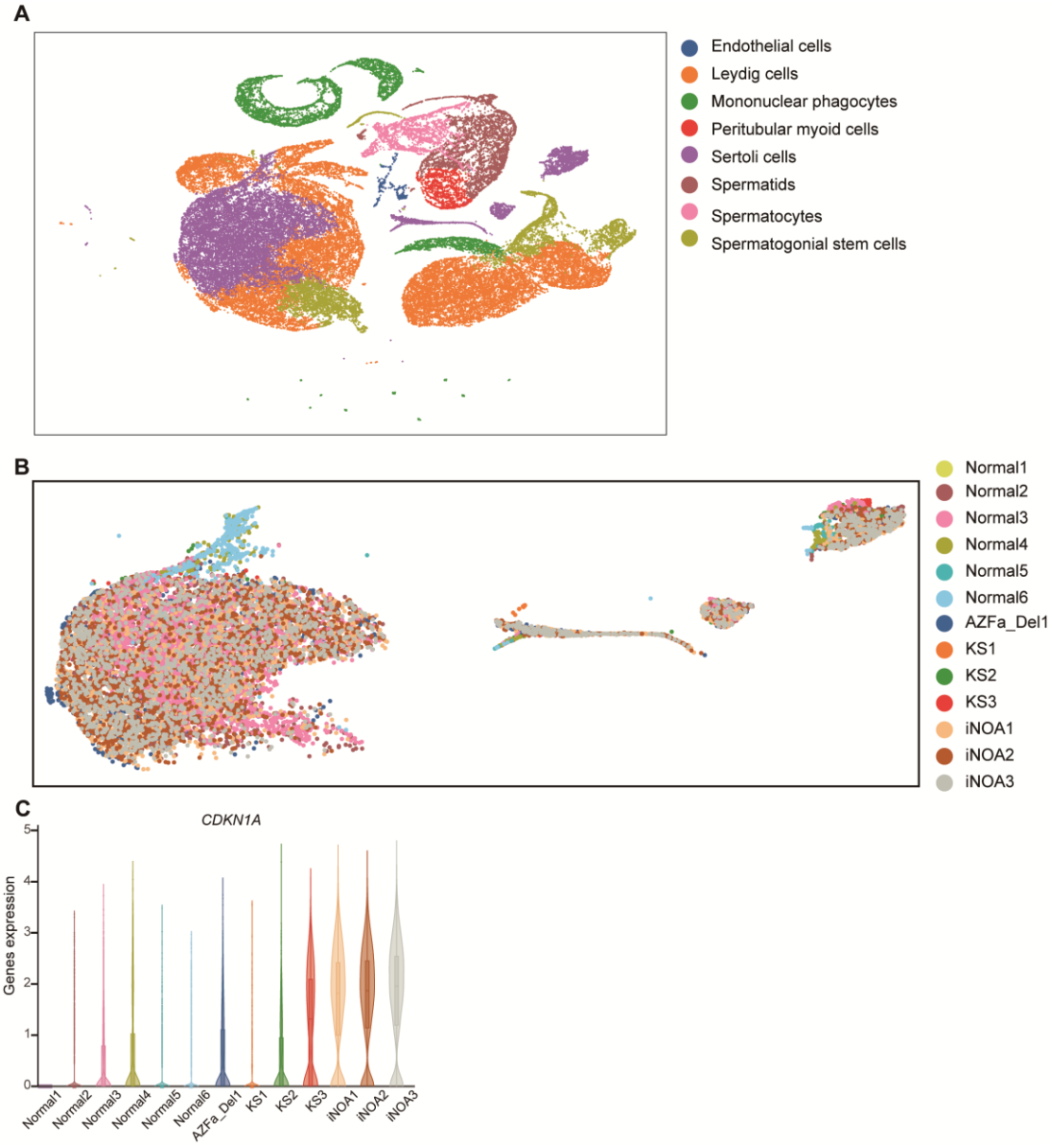
Supplementary Figure 7. CDELNs alleviated cyclophosphamide (CP)-induced male reproductive injury. (**A**) Body weight of mice throughout the experimental period ($n = 6$). (**B, C**) Periodic acid-Schiff staining (PAS staining). The ratio of round spermatids or elongated spermatids to the cross-sectional area of seminiferous tubules varies during different stages of

spermatogenesis. RS: Round spermatids, ES: Elongated spermatids. (n = 3). Groups: Ctrl, normal control; CP, cyclophosphamide model; TP, testosterone propionate (positive control); CDELNs-H, high-dose CDELNs (n = 3). (**D**) Representative immunostaining images of GATA4 in testicular sections from CP-treated mice under the interventions of CDELNs-H. (**E, F**) Following intravenous EB injection via the tail vein, plasma and testicular EB concentrations were measured in CP-treated mice following intervention with CDELNs-H (n = 4). (**G**) Transmission electron microscopy (TEM) analysis of blood-testis barrier (BTB) integrity in CP-treated mice following intervention with CDELNs-H. (**H**) Fertility assessment of CP-treated male mice following CDELNs and TP interventions, n (male mice) = 6, n (female mice) =12. (**I**) Representative flow cytometry scatter plots of sperm DNA integrity assessed by sperm chromatin structure assay. (**J**) Quantitative analysis of sperm DNA fragmentation index (DFI) in (**I**) (n = 5). (**K**) Epididymal index of CP-treated male mice following CDELNs-H and TP interventions. All data are presented as the means ± SDs. Differences between groups were assessed by one-way ANOVA followed by Dunnett's post-hoc test as appropriate, with $p < 0.05$ defined as statistically significant.

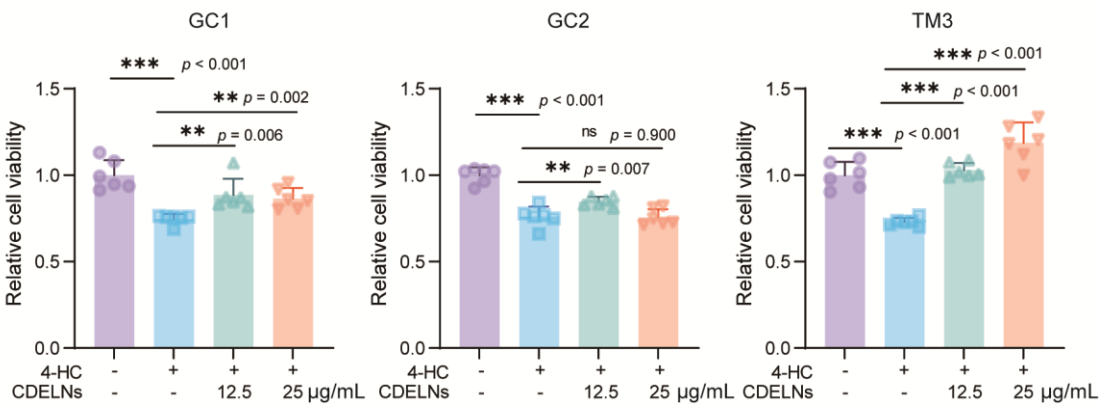


Supplementary Figure 8. Biosafety and efficacy evaluation of CDELNs in mice over a full 35-day spermatogenic cycle. (**A**) Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB) and uric acid (UA) (n = 6). (**B**) Routine blood parameters measured from diluted blood samples (n = 6). (**C**) Sperm concentration and sperm motility. Grade A: Sperms with rapid progressive motility. Grade A+B: Sperms with rapid and slow progressive motility. VCL: Curvilinear velocity. VAP: Average path velocity. VSL: Straight-line velocity. ALH: Amplitude of lateral head displacement. STR: Straightness. BCF:

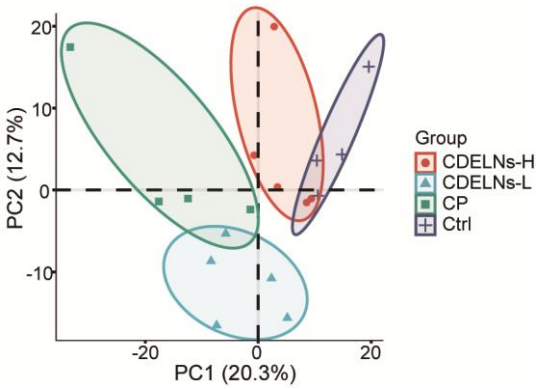
Beat-cross frequency ($n = 6$). (**D**) Representative H&E-staining images from heart, liver, spleen, lungs, kidneys, and testes of mice ($n = 6$). (**E**) Sperm concentration and motility in CP-treated mice under the interventions of CDELNs and TP ($n = 5$). All data are presented as the means ± SDs. Differences between groups were assessed by one-way ANOVA followed by Dunnett's post-hoc test or unpaired Student's *t*-test as appropriate, with $p < 0.05$ defined as statistically significant.



Supplementary Figure 9. Reanalysis of publicly available single-cell RNA-seq data from non-obstructive azoospermia (NOA) patients. (**A**) UMAP plots of all testicular cells from single-cell transcriptome profiling of normal controls and NOA patients in public database. (**B**) Analysis of normal Sertoli cells merged with iNOA, AZFa_Del, and KS Sertoli cells, in a UMAP plot with cells colored by sample type (*n* (normal) = 6, *n* (iNOA) = 3, *n* (AZFa_Del) = 1, *n* (KS) = 3). (**C**) Violin plot of the expression levels of *CDKN1A* in normal and NOA testes.



Supplementary Figure 10. CDELNs protect GC-1, GC-2, and TM3 cells from 4-HC–induced cytotoxicity. (n = 6). Data are presented as the means ± SDs. Differences between groups were assessed by one-way ANOVA followed by Dunnett's post-hoc test as appropriate, with $p < 0.05$ defined as statistically significant.



Supplementary Figure 11. Principal component analysis (PCA) of the serum metabolome from CP-treated mice following CDELNs intervention. ($n \geqslant 4$).

Supplementary Table 1. Primer sequences used for qRT-PCR

Primers	Forward (5′-3′)	Reverse (5′-3′)
Cdkn1a	CCTTGTCGCTGTCTTGCACTCTG	GCTGGTCTGCCTCCGTTTTCG
Cdkn1b	AGATACGAGTGGCAGGAGGT	ATGCCGGTCCTCAGAGTTTG
Cdkn1c	CGAGGAGCAGGACGAGAATC	GTTCTCCTGCGCAGTTCTCT
HSPG2	AGTTCCCAAGAGTCTGCACG	CTCCGGTCACAGCGATACTC
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
miR-7972	CGCTTGTCAGGCTTGTTATTCTCC	AGTGCAGGGTCCGAGGTATT
miR-6478	CCGACCTTAGCTCAGTTGGTG	AGTGCAGGGTCCGAGGTATT
miR-399e	ATTCGCCAAAGGAGAGTTGCCCT	AGTGCAGGGTCCGAGGTATT
miR-894	CGTTTCACGTCGGGTTCACC	AGTGCAGGGTCCGAGGTATT
miR-858a	CTTCGTTGTCTGTTCGACCCTG	AGTGCAGGGTCCGAGGTATT
miR-5072	ATTATCGATTCCCCAGCGGAGTC	AGTGCAGGGTCCGAGGTATT
miR-1863b	CGCAGCTCTGATACCATGTTAACTGTT	AGTGCAGGGTCCGAGGTATT
miR-159f	CGCTTGGATTGAAGGGAGCTCTA	AGTGCAGGGTCCGAGGTATT
miR-858	CTTCGTTGTCTGTTCGACCTGA	AGTGCAGGGTCCGAGGTATT
miR-1511	CCGACCTAGCTCTGATACCATGAA	AGTGCAGGGTCCGAGGTATT
miR-159b	CGCTTTGGATTGAAGGGAGCTCTC	AGTGCAGGGTCCGAGGTATT
miR-168-5p	ATATTATCGCTTGGTGCAGGTCGG	AGTGCAGGGTCCGAGGTATT
miR-171a	GCTGATTGAGCCGTGCCAATAT	AGTGCAGGGTCCGAGGTATT
miR-6300	GCGCGGTCGTTGTAGTATAGTGG	AGTGCAGGGTCCGAGGTATT
miR-159a-5p	CCGGAGCTCCTTGAAGTCCAATAG	AGTGCAGGGTCCGAGGTATT
miR-1511	GCGACCTAGCTCTGATACCATGTG	AGTGCAGGGTCCGAGGTATT
miR-159	CGTTTGGATTGAAGGGAGCTCTG	AGTGCAGGGTCCGAGGTATT
miR-171a	CGTTGAGCCGTGCCAATATCAC	AGTGCAGGGTCCGAGGTATT
miR-319c	TTGGACTGAAGGGAGCTCCT	AGTGCAGGGTCCGAGGTATT
miR-168	ATTATCGCTTGGTGCAGGTCGG	AGTGCAGGGTCCGAGGTATT
miR-858b	CGCTTCGTTGTCTGTTCGACCTTG	AGTGCAGGGTCCGAGGTATT
miR-8175	TATTATTGATCCCCGGCAACGGC	AGTGCAGGGTCCGAGGTATT
miR-166a	TCGGACCAGGCTTCATTCCTC	AGTGCAGGGTCCGAGGTATT
miR-858-5p	GGCTTTCGTTGTCTGTTCGACCTT	AGTGCAGGGTCCGAGGTATT
miR-396b-5p	CCGCTTCCACAGCTTTCTTGAACTT	AGTGCAGGGTCCGAGGTATT
miR-171b-3p	TTGAGCCGTGCCAATATCACG	AGTGCAGGGTCCGAGGTATT
miR-168a-5p	AATATCGCTTGGTGCAGGTCGG	AGTGCAGGGTCCGAGGTATT
miR-168a-3p	CCCGCCTTGCATCAACTGAAT	AGTGCAGGGTCCGAGGTATT
miR-159b-3p	CCGTTTGGATTGAAGGGAGCTCTT	AGTGCAGGGTCCGAGGTATT
miR-157d-3p	GCGGCTCTCTATGCTTCTGTCATC	AGTGCAGGGTCCGAGGTATT
miR-159	CGCTTTGGATTGAAGGGAGCTCTA	AGTGCAGGGTCCGAGGTATT
miR-396	CGCTTCCACAGCTTTCTTGAACTG	AGTGCAGGGTCCGAGGTATT
U6	CGCTTCCACAGCTTTCTTGAACTG	AACGCTTCACGAATTTGCGT

Supplementary Table 2. Sequence of miRNA mimics and anti-miR-159b-3p

Name	Forward (5′-3′)	Reverse (5′-3′)
NC mimic	UUGUACUACACAAAAGUACUG	GUACUUUUGUGUAGUACAAUU
miR-6300 mimic	GUCGUUGUAGUAUAGUGG	ACUAUACUACAACGACUU
miR-159b-3p mimic	UUUGGAUUGAAGGGAGCUCUU	GAGCUCCCUUCAAUCCAAAUU
anti-miR-159b-3p	AAGAGCUCCCUUCAAUCCAAA	

157 **Supplementary Table 3. Sequence of siRNAs**

siRNA	Forward (5′-3′)	Reverse (5′-3′)
siCtrl	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
siRNA 1-*CDKN1a*	CUGAAUAGCACUUUGGAAATT	UUUCCAAAGUGCUAUUCAGTT
siRNA 2-*CDKN1a*	GAGCAUGAAUGGAGACAGATT	UCUGUCUCCAUUCAUGCUCTT
siRNA 3-*CDKN1a*	GGUCCUUGGUGGUGAGACATT	UGUCUCACCACCAAGGACCTT
siRNA-*HSPG2*	GUACAUGGCUCAAGACUGATT	UCAGUCUUGAGCCAUGUACTT

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