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Research Article

Intrauterine Hyponutrition Reduces Fetal Testosterone Production and Postnatal Sperm Count in the Mouse

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Abstract

Background: Although intrauterine hyponutrition is regarded as a risk factor for the development of "testicular dysgenesis syndrome" (TDS) in the human, underlying mechanism(s) remain largely unknown.

Methods: To clarify the underlying mechanism(s), we fed vaginal plug-positive C57BL/6N female mice with regular food ad libitum throughout the pregnant course (control females) (C-females) or with 50% of the mean daily intake of the C-females from 6.5 dpc (calorie-restricted females) (R-females), and compared male reproductive findings between 17.5-dpc-old male mice delivered from C-females (C-fetuses) and those delivered from R-females (R-fetuses) and between 6-week-old male mice born to C-females (C-offspring) and those born to R-females (R-offspring).

Results: Compared with the C-fetuses, the R-fetuses had (1) morphologically normal external genitalia with significantly reduced anogenital distance index, (2) normal numbers of testicular component cells, and (3) significantly low intratesticular testosterone, in association with significantly reduced expressions of steroidogenic genes. Furthermore, compared with the C-offspring, the R-offspring had (1) significantly increased TUNEL-positive cells and normal numbers of other testicular component cells, (2) normal intratesticular testosterone, in association with normal expressions of steroidogenic genes, (3) significantly reduced sperm count, and normal testis weight and sperm motility, and (4) significantly altered expressions of oxidation stress-related, apoptosis-related, and spermatogenesis-related genes.

Conclusions: The results, together with the previous data including the association between testosterone deprivation and oxidative stress-evoked apoptotic activation, imply that reduced fetal testosterone production is the primary underlying factor for the development of TDS in intrauterine hyponutrition, and that TDS is included in the clinical spectrum of Developmental Origins of Health and Disease.

Key Words: intrauterine hyponutrition, testosterone production, spermatogenesis, gene expression, apoptosis

Abbreviations: AGD, anogenital distance; AGDI, anogenital distance index; AMH, anti-Müllerian hormone; C, control; Cy3, Cyanine 3; DDX4, DEAD-box helicase 4; dpc, days post coitum; FGR, fetal growth restriction; HSD3B, 3β-hydroxysteroid dehydrogenase; KO, knockout; PCNA, anti-proliferating cell nuclear antigen; PBS, phosphate-buffered saline; R, calorie-restricted females; RT-qPCR, quantitative real-time reverse transcription polymerase chain reaction; TDS, testicular dysgenesis syndrome; TUNEL, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling.

Intrauterine hyponutrition is a risk factor for the development of male reproductive dysfunction. Indeed, it has been reported that (1) fetal growth restriction (FGR) is often associated with hypospadias, cryptorchidism, and infertility in the human [1-5]; (2) maternal hyponutrition during pregnancy results in retarded testicular development, delayed puberty, and fewer Sertoli cells in the rat, lamb, and swine offspring [6-8]; and (3) maternal protein restriction during pregnancy reduces the testicular and epididymal sperm count and affects fertility in the rat offspring [9, 10]. Furthermore, it has also been reported in the mouse that (1) a large amount of glycogen is accumulated in pre-Sertoli cells immediately after the expression of the sex determining gene *Sry*, and a large amount of glucose is consumed with the activation of the testis forming gene *Sox9* [11, 12]; and (2) multiple genes involved in metabolic pathways, including the glycolysis, tricarboxylic acid cycle, and oxidative phosphorylation pathways, are strongly expressed during the differentiation of fetal Leydig cells [13]. These findings imply the critical role of appropriate nutrition and metabolism in testis development and function. It has been postulated,

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therefore, that intrauterine hyponutrition is involved in the development of "testicular dysgenesis syndrome (TDS)," which is primarily caused by adverse environmental factors in the fetal life and is associated with a variety of reproductive abnormalities including hypospadias, cryptorchidism, and infertility [4, 5].

To our knowledge, however, it remains to be clarified how intrauterine hyponutrition leads to male reproductive dysfunction. Here, we report that maternal hyponutrition during pregnancy reduces not only fetal testosterone production in association with decreased expressions of steroidogenic genes but also postnatal sperm count in association with altered expressions of spermatogenesis-, apoptosis-, and oxidation stress–related genes in the C57BL/6N mouse.

Materials and Methods

Ethics

This study was approved by the Ethics Committee for Animal Care and Use of Hamamatsu University School of Medicine (approval number 2015059), and was carried out in accordance with the institutional guidelines and the standards of humane animal care outlined in the "Guide for the Care and Use of Laboratory Animals" [14].

Primers

Primers utilized in this study are shown elsewhere (Table S1 [15]).

Experimental protocol

This study was performed using C57BL/6N mice (Japan SLC, Hamamatsu, Japan) with a pregnancy rate (the number of pregnant mice divided by that of vaginal plug-positive mice) of 65.86% and a median litter size of 7 (range 3–11). Mice were housed at a controlled temperature (23 ± 2°C) and humidity (60 ± 10%), with free access to water under a 12-hour dark/light cycle.

We performed 2 experiments in this study, using 2 groups of female mice with vaginal plugs (Fig. 1). One group of mice were fed a powdered regular chow diet (D06121301) consisting of 20% protein, 64% carbohydrate, and 16% fat (Research Diets, New Brunswick, USA) ad libitum throughout the course of pregnancy(control females) (C-females), and the other group of mice were fed 50% of the mean daily intake of the C-females from 6.5 days post coitum (dpc) (calorie-restricted females) (R-females). Noon on the day when a vaginal plug was detected was counted as 0.5 days dpc. Both C- and R-females were measured for body weight every day.

In experiment 1, C- and R-females were sacrificed using CO_2 inhalation at 17.5 dpc. Blood samples were collected from the females by cardiac puncture, and the plasma was aliquoted and stored at –30°C for biochemical studies. At the same time, we removed control fetuses (C-fetuses) and calorie-restricted fetuses (R-fetuses) from pregnant C- and R-females, respectively. C- and R-fetuses were determined for genetic sex (Figure S1 [15]), and were examined for body weight and anogenital distance (AGD) as a marker for androgen exposure during fetal life [16]. Testes were immediately excited from male C- and R-fetuses, and were utilized for subsequent analyses.

In experiment 2, pregnant C- and R-females gave birth to control offspring (C-offspring) and calorie-restricted offspring (R-offspring), respectively, at full term. After determining genetic sex (Figure S1 [15]), male mice only were utilized for the subsequent examinations. Male C-offspring were raised by their mothers, whereas male R-offspring were cross-fostered and raised by unrelated C-females. After weaning, male C- and R-offspring were fed a regular chow diet ad libitum, and were measured for weight weekly from 2 weeks of age. At 6 weeks (42 days) of age, they were sacrificed using CO_2 inhalation. Testes were immediately removed, and were utilized for subsequent analyses.

Measurements of Body Weight, Testis Weight, and Anogenital Distance

Body weights of C- and R-females/fetuses/offspring were measured with a portable balance (EK-3000i) (A&D, Tokyo, Japan), and testis weights of C- and R-offspring were measured with an analytical electronic balance (AX124) (Sartorius AG, Göttingen, Germany). AGD was measured with a Digimatic Caliper (Mitsutoyo, Kawasaki, Japan) under a EZ4 D microscope (Leica, Wetzlar, Germany). The AGD index (AGDI) was also calculated by diving the AGD by the cube root of body weight [17], to allow for comparison of AGDs between C-fetuses and R-fetuses with different body size.

Histopathological Studies

Testes fixed in Bouin solution and embedded in paraffin were cut at a 3 μm thick. Each section was mounted on SuperFrost slides (Thermo Fisher Scientific, Walthan, USA), and every tenth section was stained with hematoxylin and eosin.

The remaining sections were deparaffinized and incubated with 3% H_2O_2 in phosphate-buffered saline (PBS) to inactivate endogenous peroxidases. The slides were transferred into new blocking solution containing a rabbit polyclonal antibody against 3β-hydroxysteroid dehydrogenase (HSD3B) (1:2000) as a marker for Leydig cells [18], a goat polyclonal antibody against anti-Müllerian hormone (AMH) (1:200) (sc-6886, Santa Cruz Biotechnology, Dallas, USA) as a marker for Sertoli cells [19], a rabbit polyclonal antibody against DEAD-box helicase 4 (DDX4) (1:200) (ab13840, abcam, Cambridge, UK) as a marker for germ cells [20], or a mouse monoclonal antibody against antiproliferating cell nuclear antigen (PCNA) (1:200) (M0879, Dako, Santa Clara, USA) as a marker for proliferating cells [21], and were incubated for 30 minutes at room temperature. After washing, the slides were further incubated with corresponding secondary antibodies for 30 minutes at room temperature, and expressions were visualized using Simple Stain diaminobenzidine solution (Nichirei). HSD3B-positive cells were counted in a randomly selected field of each testis (0.17-0.26 mm^2), and AMH-positive, DDX4-positive, and PCNA-positive cells were counted in 4 or 5 selected cross-sectioned seminiferous tubules of each testis.

To evaluate apoptosis, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay [22] was performed using an Apop Tag Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Burlington, USA). In short, after incubation with proteinase K for 15 minutes at room temperature, the sections were treated with equilibration buffer, working strength TdT enzyme, stop/wash buffer, and antidigoxigenin conjugate, and TUNEL signals were visualized with diaminobenzidine staining solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). TUNEL-positive cells were examined in ~200 seminiferous tubules of each testis.

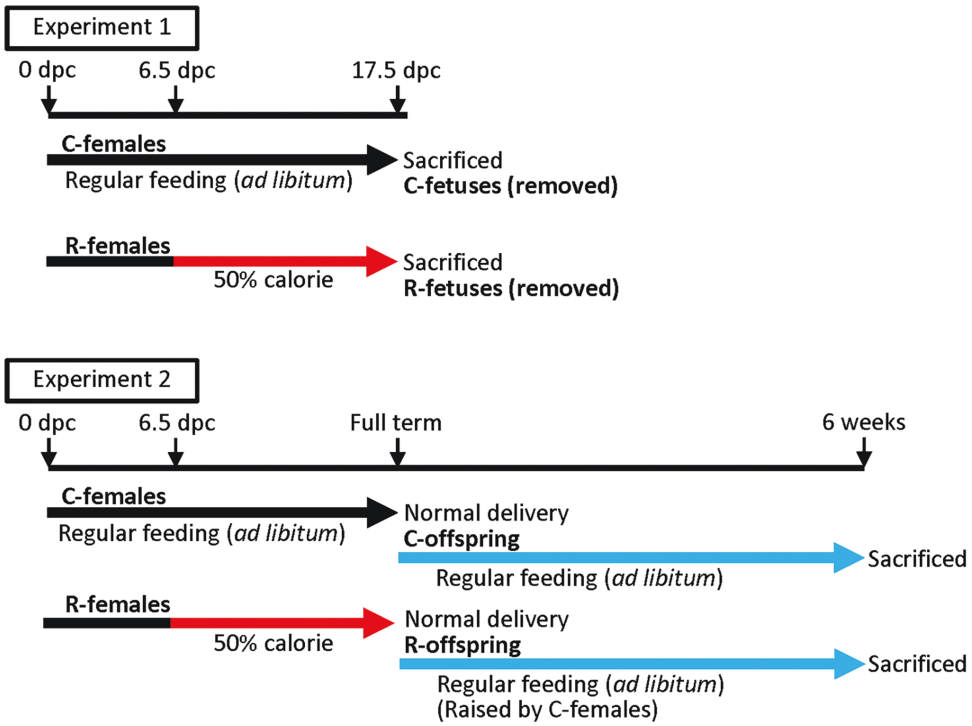

Figure 1. Experimental protocols utilized in this study.

Intratesticular testosterone measurement

Intratesticular testosterone was measured for snap-frozen testes stored at –80°C by liquid chromatography-tandem mass spectrometry, using the previously described method [23]. In brief, the testis samples were minced and homogenized in 1 mL of 100% methanol using a Bioruptor UCD-250 (BM Equipment, Tokyo, Japan), and 10 ng of d3-testosterone (Sigma-Aldrich, St. Louis, USA) was added as an internal standard. Testosterone extracts were obtained by centrifugation at 1000*g* for 2 minutes at room temperature. Calibration standards covering the range of 0.01 to 100 ng/mL were prepared using standard testosterone (0.01, 0.1, 0.5, 1,5, 10, 25, 50, and 100 ng/mL), alongside the internal standard (10 ng). The samples were then filtered using a membrane filter (pore size, 0.22 µm) and injected into a QTRAP liquid chromatography-tandem mass spectrometry system (SCIEX, Framingham, USA) for the quantification of testosterone.

Sperm Count and Motility Analyses

Epididymal sperms were counted as described previously [24]. In brief, after removing the epididymis from testis, cauda epididymis was detached from the junction of the vas deferens and epididymal corpus, and was weighed with an AX124. The cauda epididymis was dipped in 2 mL of prewarmed PBS, and the epididymal canal was cut with ophthalmologic scissors. After incubation for 15 minutes at 37°C, the suspension was diluted to 1:4 in PBS and incubated for 1 minute at 60°C to kill sperm. The number of sperm was counted for 10 µL of the suspension on a cell counting plate (AGC TECHNO GLASS, Shizuoka, Japan) using a Leica DMi1 (Leica Microsystems, Wetzlar, Germany) and was expressed on a per-mass basis.

Sperm motility was analyzed as described previously [25]. In short, the excised cauda epididymis was minced in modified Tyrode's albumin/lactate/pyruvate medium. The tissue fragments were then incubated in a 5% CO_2 incubator at 37°C for 10 minutes, and the medium was loaded onto a Leja Standard Count 2-Chamber Slide (Leja, Nieuw-Vennep, The Netherlands). Sperm motility was observed with an IX81N-22TFL/PH-2 microscope (Olympus, Tokyo, Japan) equipped with a LUCPlanFLN 20×/0.45 objective lens (Olympus), and was recorded at 200 frames/second using an acA1300-200uc camera and Pylon Viewer software (Basler, Ahrensburg Germany). Motility analyses were performed using the Particle Tracker plugin of ImageJ software (National Institutes of Health, Bethesda, USA).

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction Analysis

RNA was isolated from snap-frozen testes stored at –80°C in RNAlater™ Stabilization Solution (Thermo Fisher Scientific), using an RNeasy Micro Kit (Qiagen, Hulsterweg, Netherlands) or an RNeasy Mini Kit (Qiagen). Pooled testes from litter mice were utilized in male C- and R-fetuses because of the tiny testis size, whereas single testes were utilized in male C- and R-offspring. The concentration and integrity of RNA samples were assayed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and an Agilent 4200 TapeStation (Agilent, Santa Clara, CA, USA). RT was performed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan), and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) was performed using Thunderbird SYBR qPCR Mix (Toyobo) in the StepOnePlus system, using Software v2.2.2 (Thermo Fisher Scientific). The relative expression was calculated using the $\Delta\Delta C_t$ method and normalized to that of *Gapdh* used as the reference gene.

Microarray Analysis

Microarray analysis was performed for RNA samples obtained from the testes of C- and R-offspring, using single-color SurePrint G3 mouse GE 8 × 60 K v2 microarrays (Agilent Technologies, Santa Clara, CA, USA). The RNA was

amplified and labeled with Cyanine 3 (Cy3) using a Low Input Quick Amp Labeling Kit (Agilent Technologies), and purified using RNeasy columns (Qiagen Valencia, CA, USA). The cRNA quantity and cyanine incorporation were assessed using the NanoDrop ND-1000 spectrophotometer and an Agilent Bioanalyzer. Then, the Cy3-labeled cRNA was fragmented and hybridized to the microarray. After washing, the microarrays were scanned on an Agilent SureScan Microarray Scanner (G2600D) using the 1-color scan setting for 8 × 60 K. The scanned images were analyzed with Feature Extraction Software 12.0.3.1 (Agilent Technologies) using the default parameters. Normalization was performed using the global scaling method. We extracted genes satisfying the combined criterion for true positive expression differences of a fold difference of >1.5 or <0.66 ($\log_2$ ratio > |0.58|) and a false discovery rate (q value) of <0.05. The microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE186345 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE186345).

Statistical Analysis

The variables were expressed as the mean ± SE. For statistical analyses, we first examined normality by the chi-squared test. For variables following normality, we employed Student's *t*-test for 2 groups with similar variances and Welch's t-test for 2 groups with different variances, after comparing variances by the F-test. For variables not following normality, we utilized Mann-Whitney's U-test. Statistical significance of the frequency was analyzed by the Fisher's exact probability test. $P < .05$ was considered statistically significant.

Results

Pregnancy and Delivery

We utilized a total of 178 female mice with vaginal plugs in this study: 30 C-females and 33 R-females in experiment 1, and 49 C-females and 66 R-females in experiment 2 (Table 1). All R-females survived under 50% calorie restriction, although body weight became significantly lower in R-females than in C-females from 11.5 dpc (Fig. 2A). In total (experiments 1 + 2), the pregnancy rate was significantly lower and litter size was significantly smaller in R-females than in C-females, whereas the sex ratio of the fetuses and offspring was similar between C-females and R-females (Table 1).

Biochemical Data of Pregnant C- and R-females

The plasma values of lipids (triglyceride, total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol) and glucose were significantly lower in the pregnant R-females examined than in the pregnant C-females examined, whereas those of total protein and albumin were similar between the C-females and the R-females (Table 2).

Growth and AGD/AGDI in R-fetuses/offspring

The body weight was significantly reduced in R-fetuses compared with C-fetuses of both sexes (Table 3), and became comparable between the male C-offspring and the male R-offspring by 2 weeks of age (Fig. 2B). The AGD was significantly shorter in R-fetuses than in C-fetuses of both sexes, and the AGDI was significantly shorter in male R-fetuses than in male C-fetuses and similar between female C-fetuses and female R-fetuses (Table 3). There were no apparent structural abnormalities of external genitalia in male R-fetuses and R-offspring.

Histopathological Studies

Histological and immunohistochemical findings were grossly similar between the C-fetuses and the R-fetuses and between the C-offspring and the R-offspring (Fig. 3), with comparable numbers of HSD3B-, AMH-, DDX4-, and PNCA-positive cells (Table 4). Notably, however, while TUNEL-positive cells were barely identified in the C-fetuses and R-fetuses, the percentage of tubules containing TUNEL-positive cells was significantly larger in R-offspring than in C-offspring, and the number of TUNEL-positive cells per 100 tubules was, though not significant, apparently high in the R-offspring (Table 4).

Table 1. Data on pregnancy and delivery

	C-females	R-females	*P* value
Experiment 1			
Pregnancy rate[a]	20/30 (67%)	13/33 (34%)	**.044**
Litter size (n/dam)	6.45 ± 0.34	5.77 ± 0.56	.28
Sex ratio in fetuses (M:F)	79:50	38:37	.15
Experiment 2			
Pregnancy rate[a]	29/49 (59%)	31/66 (47%)	.26
Litter size (n/dam)	6.96 ± 0.33[b]	5.10 ± 0.58[b]	**.0045**
Sex ratio in newborns (M:F)	102:93[a]	58:49[a]	.81
Experiments 1 + 2			
Pregnancy rate[a]	49/79 (62%)	44/99 (44%)	**.024**
Litter size (n/dam)	6.75 ± 0.24	5.35 ± 0.41	**.0026**
Sex ratio (M:F)	179:143	96:86	.58

Reference data: the pregnancy rate, 65.86%; and the litter size, median 7 (range 3-11) (Japan SLC, Hamamatsu, Japan). *P* values < 0.05 are boldfaced.
Abbreviations: C, control; R, calorie-restricted; M, male; and F, female.
[a]The number of female mice confirmed to be pregnant at 17.5 days post coitum (experiment 1) or at term (experiment 2) divided by the number of females with vaginal plugs.
[b]These data have been obtained from after excluding 2 C-females and 8 R-females which showed cannibalism.

Testosterone Production and Expression Profile of Steroidogenic Genes

The results are shown in Fig. 4, and the *P* values for gene expressions levels are summarized elsewhere (Table S2 [15]). The intratesticular testosterone value was significantly lower in R-fetuses than in C-fetuses at 17.5 dpc. Consistent with this, RT-qPCR analysis showed that expression of *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, and *Hsd17b3* involved in steroidogenesis were significantly lower in the testis samples of R-fetuses than in those of C-fetuses. In addition, expressions of *Nr5a1* (alias, *Sf-1* and *Ad4bp*) and *Insl3* were also significantly lower in R-fetuses than in C-fetuses, whereas those of *Sox9* and *Amh* were similar between the C-fetuses and the R-fetuses. By contrast, the intratesticular testosterone value was similar between the R-offspring and the C-offspring at 6 weeks of age. In accordance with this, RT-qPCR revealed that expressions of *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Hsd17b3 Nr5a1*, and *Insl3* were comparable between testis samples of the R-offspring and C-offspring, as were those of *Sox9* and *Amh*.

Spermatogenic Function and Expression Profile of Relevant Genes

The results are shown in Fig. 5, and the *P* values for gene expressions levels are summarized elsewhere (Table S2 [15]). The sperm count was significantly lower in R-offspring than in C-offspring (Figure S2 [15]), while the testicular size and sperm motility were comparable between R-offspring and C-offspring. Microarray analysis revealed 1082 upregulated and 446 downregulated genes in the testis samples of R-offspring compared with those of C-offspring, including 67 upregulated and 26 downregulated genes linked to "spermatogenesis", 20 upregulated and 14 downregulated genes linked to "germ cell", 8 upregulated and 8 downregulated genes linked to "Sertoli cell", and 1 upregulated and 4 downregulated genes linked to "Leydig cell" in the NCBI Entrez Gene database (https://www.ncbi.nlm.nih.gov/) (Table S3 [15]). Subsequently, we selected 2 upregulated and 8 downregulated genes which have been reported to be relevant to spermatogenic function, and validated their expression levels by RT-qPCR. Consequently, RT-qPCR confirmed significantly upregulated expressions of *Notch1* and *Esr2* and significantly downregulated expressions of *Amhr2*, *Dazl*, *Hormad1*, *Nr0b1* (alias, *Dax1*), *Gja1*, *Stra8*, and *Inha* in the R-offspring; the *Defb19* expression was, though reduced in the R-offspring, not significantly different between the R-offspring and the C-offspring.

Furthermore, we also went over the expression levels of previously known apoptosis-related genes, revealing significantly increased expression of *Bcl2l1* and significantly decreased expressions of *Bax*, *Bid*, and *Casp6* in the R-offspring. In addition, expression analyses of genes involved in apoptosis-evoking events showed significantly altered expressions of oxidative stress–related genes such as increased expression of *Nox4* and significantly decreased expressions of *Gstp1*, *Gpx1*, *Prdx1*, and *Prdx2* in the R-offspring.

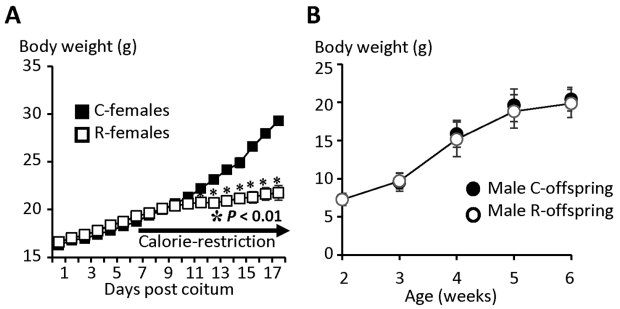

Figure 2. Graphic representation of body weights. (A) Body weights of control (C) and calorie-restricted (R) female mice during pregnancy. (B) Body weights of male control (C) and calorie-restricted (R) offspring mice.

Discussion

We performed 50% calorie restriction for R-females from 6.5 dpc to examine the effects of intrauterine hyponutrition on male reproductive functions in the C57BL/6N mouse. Although the caloric restriction did not affect survival of the R-females and the sex ratio of the R-fetuses and R-offspring, it apparently reduced the pregnancy rate and litter size. The results, in conjunction with the marked weight reduction of R-females and R-fetuses, imply that the caloric restriction employed in this study is severe enough to examine the effects of hyponutrition in the R-fetuses and R-offspring. In addition, the biochemical data of the R-females suggest that lipid and carbohydrate metabolism is more susceptible to caloric restriction than protein metabolism, and the catch-up growth of the R-offspring under normal nutrition is consistent with the growth failure of the R-fetuses being caused by intrauterine environmental factors.

Intratesticular testosterone was significantly lower in male R-fetuses than in male C-fetuses, in association with reduced expressions of steroidogenic genes. This is consistent with the AGDI being significantly lower in male R-fetuses than in male C-fetuses, because AGDI reflects the degree of androgen action after compensation for the body weights [17]. Indeed, since the AGDI, but not AGD, was similar between female C-fetuses and

Table 2. Blood biochemical values of pregnant C- and R-Females at 17.5 dpc

	C-females (n = 8)	R-females (n = 6)	*P* value
Triglyceride (mg/dL)	65.0 ± 5.56	25.7 ± 3.34	**.0019**
Total cholesterol (mg/dL)	60.0 ± 3.38	38.0 ± 3.14	**.0019**
LDL cholesterol (mg/dL)	5.75 ± 0.67	4.00 ± 1.06	**.049**
HDL cholesterol (mg/dL)	25.4 ± 1.46	18.2 ± 1.78	**.0083**
Glucose (mg/dL)	206 ± 13.8	145 ± 13.9	**.010**
Total protein (g/dL)	5.41 ± 0.37	5.65 ± 0.24	.37
Albumin (g/dL)	3.69 ± 0.08	3.87 ± 0.12	.22

P values < 0.05 are boldfaced.
Abbreviations: C, control; R, calorie-restricted; LDL, low-density lipoprotein; HDL, high-density lipoprotein; and dpc, days post coitum.

Table 3. Body weight and AGD/AGDI of C- and R-fetuses at 17.5 dpc

		C-fetuses	R-fetuses	*P* value
Weight (g)	Male	0.82 ± 0.01 (n = 73)	0.61 ± 0.02 (n = 33)	**1.7×10^{-11}**
	Female	0.88 ± 0.03 (n = 46)	0.59 ± 0.02 (n = 29)	**6.4×10^{-10}**
AGD (mm)	Male	1.37 ± 0.03 (n = 73)	1.14 ± 0.33 (n = 33)	**.00015**
	Female	0.74 ± 0.02 (n = 46)	0.61 ± 0.02 (n = 29)	**.00034**
AGDI (mm/g^3)	Male	1.46 ± 0.03 (n = 73)	1.34 ± 0.06 (n = 33)	**.042**
	Female	0.78 ± 0.02 (n = 46)	0.73 ± 0.03 (n = 29)	.20

P values < 0.05 are boldfaced.
Abbreviations: C, control; R, calorie-restricted; AGD, anogenital distance; AGDI, anogenital distance index; dpc, days post coitum.

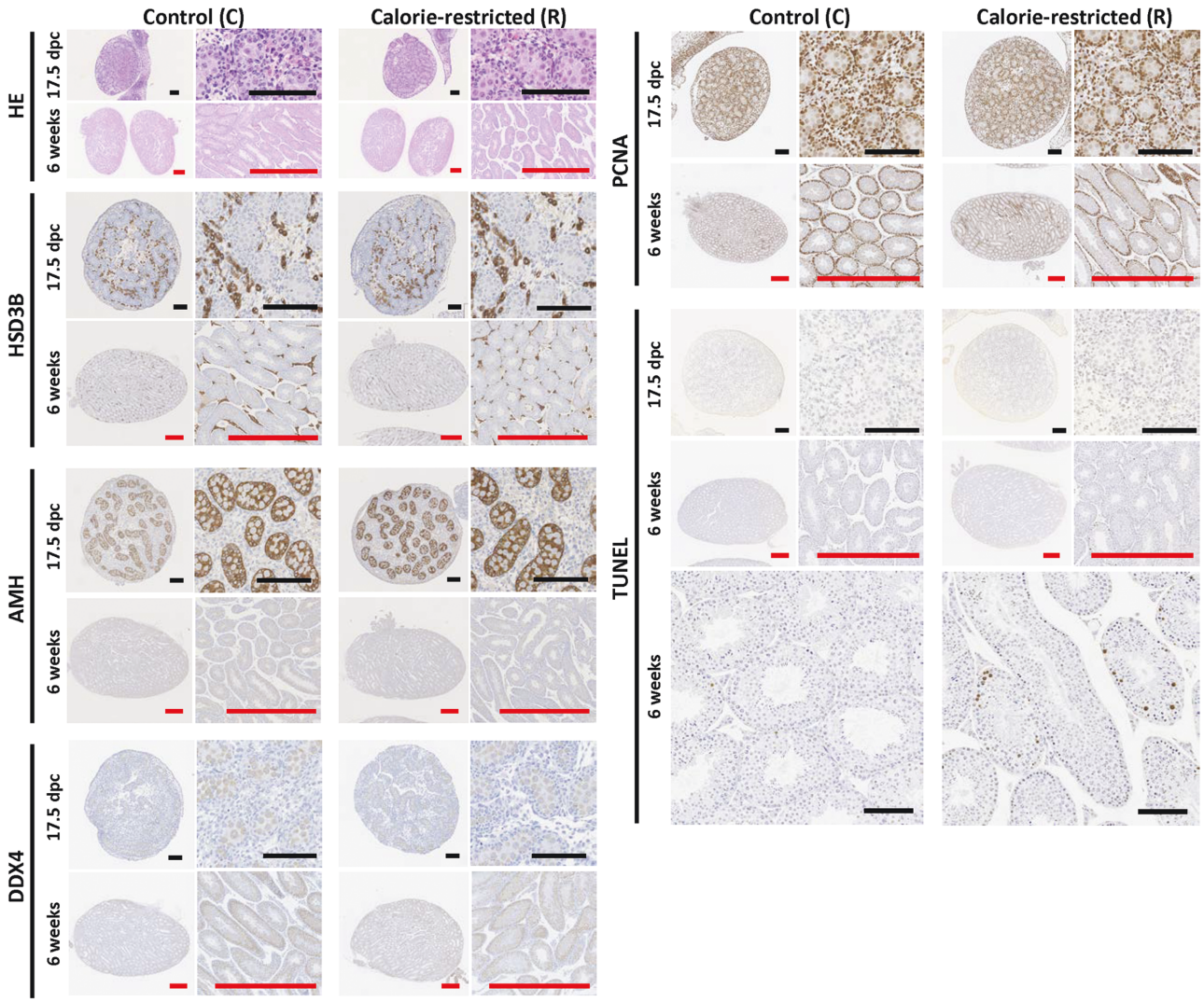

Figure 3. Low-power-field and high-power-field histopathological findings. Hematoxylin and eosin stained histological findings are similar between C-fetuses and R-fetuses at 17.5 dpc and between C-offspring and R-offspring at 6 weeks of age, as are immunohistochemical findings of HSD3B-positive Leydig cells, AMH-positive Sertoli cells, DDX4-positive germ cells, and PNCA-positive proliferating cells. However, TUNEL-positive apoptotic cells are, though barely detected in C-fetuses and R-fetuses, apparently more frequent in R-offspring than in C-offspring at 6 weeks. The black and red bars indicate 100 μm and 1 mm, respectively.

R-fetuses, the decreased AGDI in male R-fetuses would be inexplicable by the growth failure. In this regard, since the testicular histopathological findings argue against hypoplasia of fetal steroidogenic cells (fetal Leydig cells for *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b1*, and fetal Sertoli cells for *Hsd17b3*) [26], it is inferred that intrauterine hyponutrition compromises fetal testosterone production primarily via hypofunction of fetal steroidogenic cells.

Although the underlying mechanism(s) leading to reduced testosterone production in male R-fetuses remains to be clarified, several matters are worth pointing out. First, testis development takes place around 12.5 dpc [27], whereas

Table 4. Cell numbers of C-and R-fetuses at 17.5 dpc and C-and R-offspring at 6 weeks of age

		C	R	*P* value
HSD3B-positive cells	Fetuses	1662 ± 63 (n = 3)	1511 ± 292 (n = 3)	.63
(No. per mm^2)	Offspring	340 ± 42 (n = 3)	397 ± 62 (n = 3)	.48
AMH-positive cells	Fetuses	22.9 ± 2.9 (n = 3)	20.5 ± 0.4 (n = 3)	.44
(No. per tubule)	Offspring	20.7 ± 0.3 (n = 3)	19.0 ± 1.0 (n = 3)	.19
DDX4-positive cells	Fetuses	7.19 ± 0.6 (n = 3)	7.94 ± 0.5 (n = 3)	.36
(No. per tubule)	Offspring	179 ± 16 (n = 3)	210 ± 3 (n = 3)	.14
PCNA-positive cells	Fetuses	20.5 ± 1.4 (n = 3)	20.1 ± 1.1 (n = 3)	.83
(No. per tubule)	Offspring	61.3 ± 3.9 (n = 3)	72.5 ± 4.0 (n = 3)	.11
TUNEL-positive tubules	Fetuses	Not observed	Not observed	—
(% per 100 tubules)	Offspring	19.6 ± 1.6 (n = 4)	31.8 ± 2.1 (n = 4)	**.021**
TUNEL-positive cells	Fetuses	Not observed	Not observed	—
(No. per 100 tubules)	Offspring	66.6 ± 11.6 (n = 4)	124.2 ± 22.2 (n = 4)	.083

P values < 0.05 are boldfaced.
Abbreviations: C, control; R, calorie restricted; No., number; dpc, days post coitum.

intratesticular testosterone production is highest around 18.5 dpc in the mouse [28]. Thus, intrauterine hyponutrition, though it would have more or less compromised expressions of testis development genes around the critical period for testis development when a large amount of glucose is consumed [12], would have primarily affected expression of steroidogenic genes at 17.5 dpc when steroidogenic metabolism would be active with strong expression of multiple genes involved in metabolic pathways, including the glycolysis, tricarboxylic acid cycle, and oxidative phosphorylation [13]. Second, cholesterol as the substrate of steroidogenesis was reduced in the R-females. If cholesterol was also decreased in the R-fetuses, this would be involved in reduced testosterone production, as has been shown in Smith–Lemli–Opitz syndrome [29]. Lastly, *Nr5a1* expression was reduced in male R-fetuses. In this regard, *Nr5a1* upregulates the expression of steroidogenic genes as well as *Insl3* [30]. Furthermore, *Nr5a1* not only regulates de novo cholesterol production from acetyl-CoA [31] but also maintains intracellular adenosine triphosphate and nicotinamide adenine dinucleotide phosphate concentrations required for de novo steroid biosynthesis from acetyl-CoA [32, 33]. Thus, compromised *Nr5a1* expression would have played a certain role in the development of reduced testosterone production, as well as decreased *Insl3* expression. While expression of *Sox9* and *Amh* was not significantly reduced, despite their expression being upregulated by *Nr5a1* [30], this may be due to *Sox9* and *Amh* expression being controlled by multiple genes, in cooperation with *Nr5a1* [34, 35]. It is inferred that these factors would have exerted a synergic effect, resulting in the hypofunction of steroidogenic cells.

The R-fetuses, though they had a low AGDI, exhibited no structural abnormality of the external genitalia such as hypospadias, in contrast to the high prevalence of male genital abnormality such as hypospadias in patients born with FGR [1-5]. This would primarily be due to 2 factors. One factor is species difference. In humans, the urethra is constructed at 12 to 15 weeks of gestation when intratesticular testosterone is high [36], whereas in the mouse, the urethra is formed at 15.5 to 17.5 dpc before the intratesticular testosterone becomes high at 18.5 dpc [28, 37]. Thus, murine urethra may be generated with a relatively small amount of testosterone. The other factor is selection bias in humans. In contrast to the mouse studies that can be performed without selection for phenotype, human studies are usually carried out for patients with abnormal phenotype. Such selection bias would underlie the apparently high frequency of male genital abnormality in humans.

Intratesticular testosterone was recovered at 6 weeks of age, in association with the normal expression levels of *Nr5a1* and steroidogenic genes. This implies that testosterone production can be normalized with pertinent postnatal nutrition. Furthermore, this may explain why underlying factors remain largely unknown for under-masculinization such as hypospadias in patients born with FGR [1], because such patients may have compromised testosterone production primarily during fetal life due to extrinsic intrauterine hyponutrition rather than intrinsic endocrine dysfunction.

The sperm count was significantly reduced in male R-offspring at 6 weeks of age, although the intratesticular testosterone value, testis weight, and sperm motility were normal. Considering the duration of spermatogenesis and movement from the testis to the cauda epididymis [38, 39], the analyzed sperm would have been produced shortly after birth when intratesticular testosterone is assumed to still be low. In this regard, it has been reported that testosterone deprivation leads to germ cell apoptosis through several deleterious effects including oxidative stress [40], and this study revealed apparently more increased TUNEL-positive cells and oxidative stress (ie, increased expression of *Nox4* mediating oxidative stress and decreased expression of *Gstp1*, *Gpx1*, *Prdx1*, and *Prdx2* with anti-oxidative stress [41-45]) in R-offspring, although the data were obtained at 6 weeks of age. It is possible, therefore, that decreased intratesticular testosterone production around the time of initial spermatogenesis has reduced sperm count via enhanced oxidative stress and the resultant occurrence of apoptosis. While microarray analysis at 6 weeks of age indicated upregulation of anti-apoptotic *Bcl2l1* and downregulation of pro-apoptotic *Bax* and *Bid* and apoptosis-related protease *Casp6* [46-49], this may be explained as the protective responses against the occurrence of apoptosis. However, since sperm production is a complicated phenomenon, multiple factors would remain undetected in the development of reduced sperm count in the R-offspring.

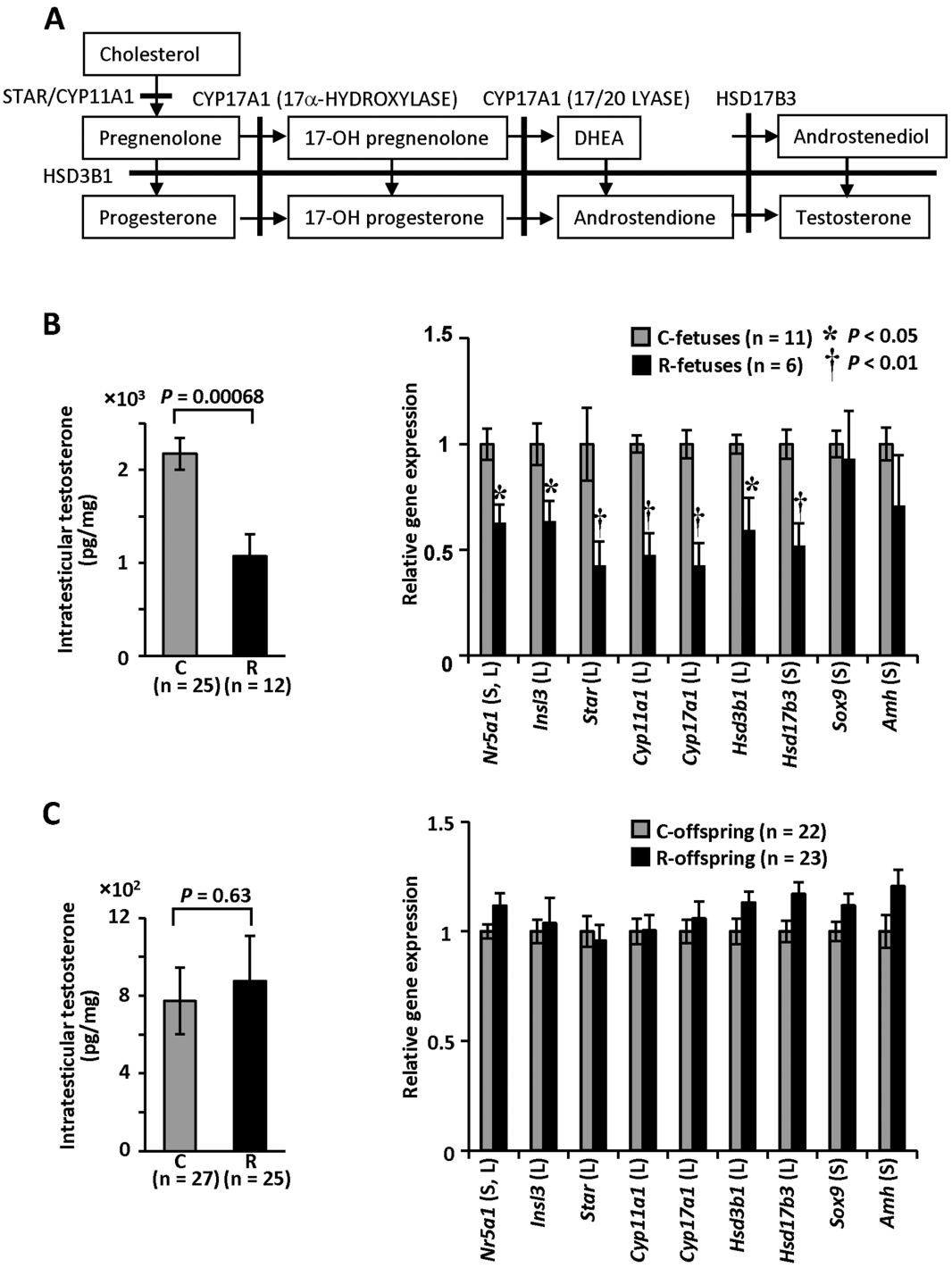

Figure 4. Testosterone production-related findings. C, control; R, calorie-restricted; L, Leydig cell; and S, Sertoli cell. (A) Steroidogenic pathway from cholesterol to testosterone and enzymes involved in each conversion. (B) Intratesticular testosterone value and expression profile of steroidogenic genes obtained by RT-qPCR in male C- and R-fetuses at 17.5 dpc. (C) Intratesticular testosterone value and expression profile of steroidogenic genes obtained by RT-qPCR in male C- and R-offspring at 6 weeks of age.

The gene expression patterns at 6 weeks of age are noteworthy in terms of spermatogenic function of the R-offspring. Indeed, *Notch1* and *Esr2* were shown to be upregulated by both microarray and RT-qPCR analyses, and it has been reported that (1) *Notch1* gain of function in germ cells causes spermatogenic failure in mice [50]; and (2) activation of *Esr2* causes spermatocyte apoptosis and spermiation failure [51]. Similarly, *Amhr2*, *Dazl*, *Hormad1*, *Nr0b1*, *Gja1*, *Stra8*, and *Inha* were revealed to be downregulated by both microarray and RT-qPCR analyses, and it has been reported that (1) *Amhr2* is expressed in spermatocytes as well as Sertoli cells, and *AMHR2* mutations are frequently associated with infertility in humans [52, 53]; (2) *DAZL* variants are associated with spermatogenic failure in humans, and *Dazl* knockout (KO) male mice show spermatogenic failure [54, 55]; (3) *Hormad1* is involved in synaptonemal complex formation, and *Hormad1* KO male and female mice are infertile [56, 57]; (4) *Nr0b1* plays a critical role in adrenal and reproductive

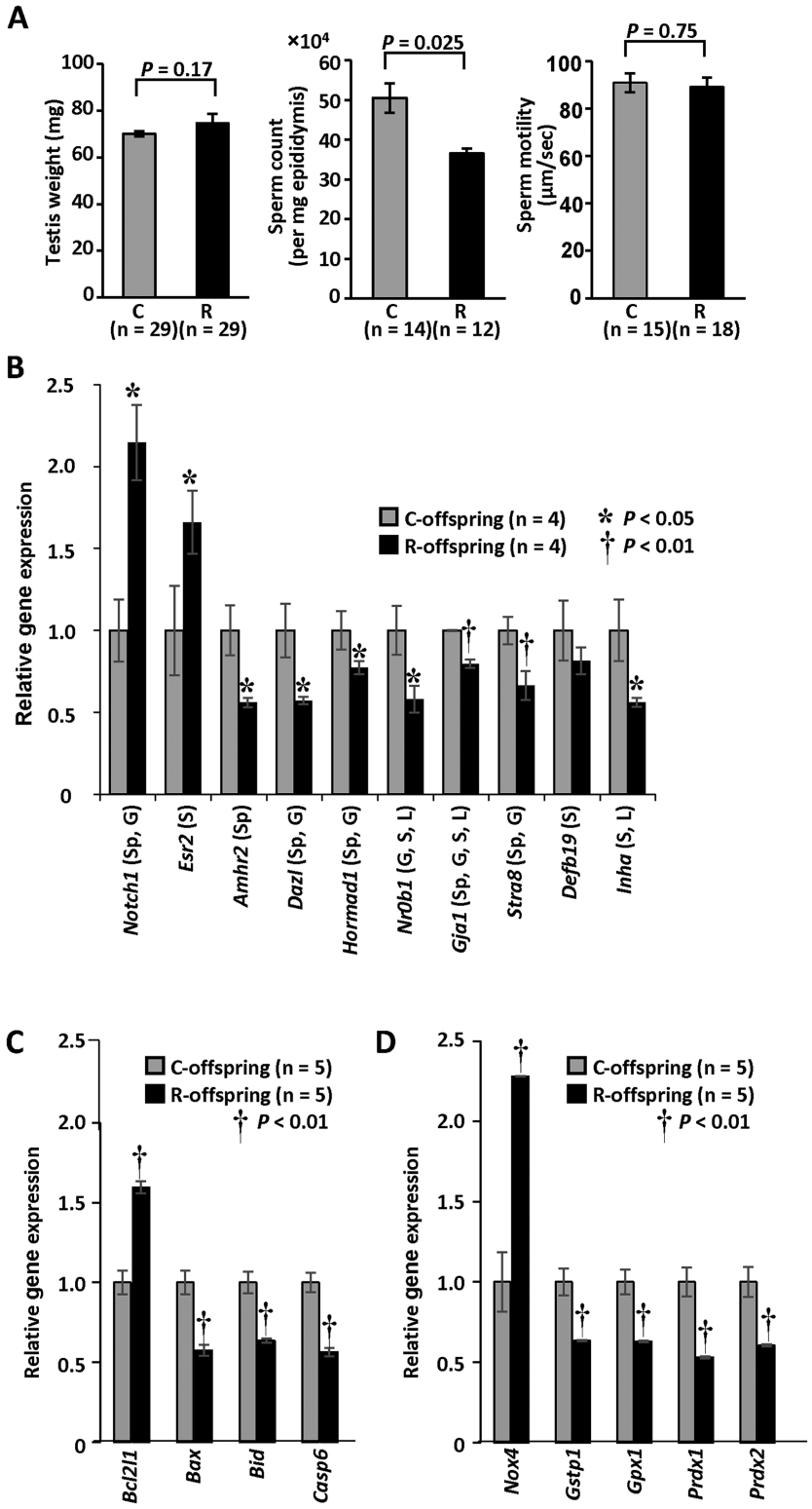

Figure 5. Sperm formation-related findings in male control (C) and calorie-restricted (R) offspring at 6 weeks of age. (A) Comparisons of testis weight, sperm count, and sperm motility. (B) Expression profile of spermatogenesis (Sp)-related, germ cell (G)-related, Sertoli-cell (S) related, and Leydig cell (L)-related genes obtained by RT-qPCR. (C) Expression profile of apoptosis-related genes obtained by microarray. (D) Expression profile of oxidation stress-related genes obtained by microarray.

development, and *Nr0b1* KO male mice show progressive spermatogenic dysfunction and resultant infertility [58, 59]; (5) *Gja1* is a testicular gap junction protein bridging Sertoli cells and germ cells, and *Gja1* KO male mice are infertile because of maturation arrest [60, 61]; (6) *Stra8* is specifically expressed in germ cells, and *Stra8* KO male and female mice are incapable of initiating meiosis [62]; and (7) *Inha* is strongly expressed in Sertoli cells, and *Inha* heterozygous

KO male mice have reduced spermatogenic activity [63, 64]. These findings imply that intrauterine hyponutrition leads to altered expressions of multiple genes which could exert an accumulative deleterious effect on spermatogenesis, although the underlying mechanism(s) including the relevance of compromised fetal testosterone production and activated apoptosis remains to be clarified, as well as the fertility capacity in a later age.

Two matters are worth pointing out in this study. First, the results imply that reduced fetal testosterone production underlies the development of TDS in patients born with FGR [4, 5]. Indeed, male R-fetuses had compromised testosterone production, although they lacked cryptorchidism and hypospadias. Similarly, male R-offspring had reduced sperm count which could be ascribed to reduced fetal testosterone production, although it remains to be clarified whether they have compromised fertility. Thus, this study may provide for the first time a missing link between FGR and TDS. Second, this study may expand the spectrum of the Developmental Origins of Health and Disease [65]. According to the Developmental Origins of Health and Disease, exposure to certain environmental insults during critical periods of development and growth can have significant consequences on an individual's short- and long-term health. This concept is consistent with the development of TDS in human subjects born with FGR, as well as the occurrence of metabolic disease in later life.

In summary, we examined the effects of intrauterine hyponutrition on male reproductive functions in the C57BL/6N mouse. The results argue for the association between FGR and male reproductive dysfunction.

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Author Contributions

Y.F., H.I., M.F., K.M., and T.O. conceived and designed the study. H.O., A.K., I.Y., Y.K-F., M.M., and T.B., performed the experiments. Y.F., H.O., A.K., I.Y., M.F., K.M., and T.O. analyzed the data. Y.F. and T.O. wrote the paper, with an input from M.F. and K.M.

Disclosure Summary

The authors have nothing to disclose.

Data Availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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