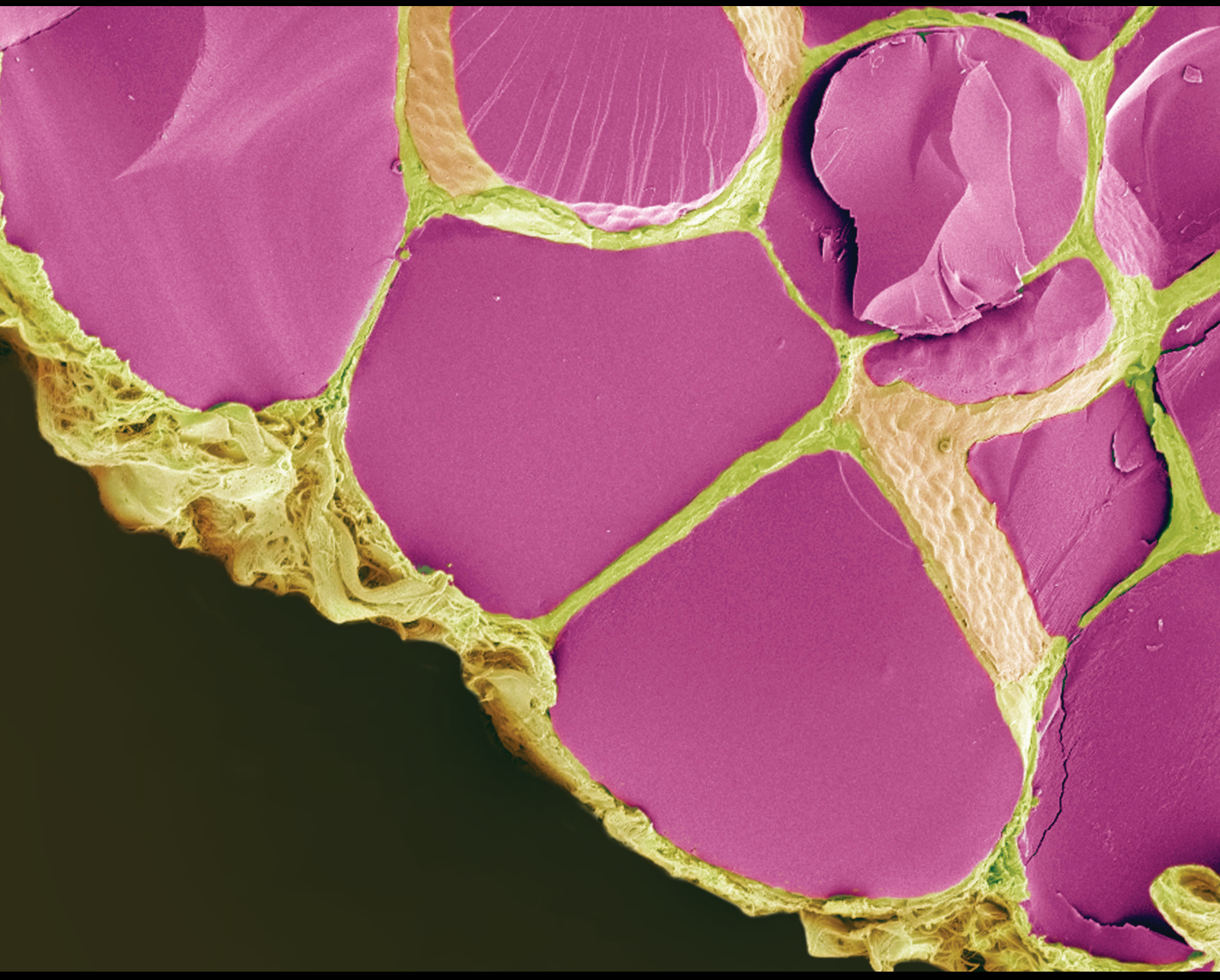
International Journal of Endocrinology

Endocrine Responses Shaped by Ageing, Diet, and Environmental Endocrine Disruptors

Lead Guest Editor: Rosanna Chianese
Guest Editors: Renata S. Tavares and Matilde Cescon



Endocrine Responses Shaped by Ageing, Diet, and Environmental Endocrine Disruptors

International Journal of Endocrinology

Endocrine Responses Shaped by Ageing, Diet, and Environmental Endocrine Disruptors

Lead Guest Editor: Rosanna Chianese
Guest Editors: Renata S. Tavares and Matilde Cescon

Chief Editor

Academic Editors

Contents

Quantitative Analysis of the Proteome and the Succinylome in the Thyroid Tissue of High-Fat Diet-Induced Hypothyroxinemia in Rats
Baoxiang Hu, Meng Zhao, Dandan Luo, Chunxiao Yu, Shanshan Shao, Lifang Zhao, Yashuang Yang, Xiaohan Zhang, Jiajun Zhao, and Ling Gao
Research Article (15 pages), Article ID 3240198, Volume 2020 (2020)

Epigenetic Modifications due to Environment, Ageing, Nutrition, and Endocrine Disrupting Chemicals and Their Effects on the Endocrine System
Elizabeth C. Plunk and Sean M. Richards
Review Article (11 pages), Article ID 9251980, Volume 2020 (2020)

Selenium Supplementation, Body Mass Composition, and Leptin Levels in Patients with Obesity on a Balanced Mildly Hypocaloric Diet: A Pilot Study
Elisabetta Cavedon, Jacopo Manso, Isabella Negro, Simona Censi, Roberto Serra, Luca Busetto, Roberto Vettor, Mario Plebani, Raffaele Pezzani, Davide Nacamulli, and Caterina Mian
Research Article (7 pages), Article ID 4802739, Volume 2020 (2020)

Older Age Is Associated with Decreased Levels of VDR, CYP27B1, and CYP24A1 and Increased Levels of PTH in Human Parathyroid Glands
Yi Jiang, Liyan Liao, Jina Li, Larry Wang, and Zhongjian Xie
Research Article (6 pages), Article ID 7257913, Volume 2020 (2020)

Fetal-Perinatal Exposure to Bisphenol-A Affects Quality of Spermatozoa in Adulthood Mouse
Teresa Chioccarelli, Francesco Manfrevola, Marina Migliaccio, Lucia Altucci, Veronica Porreca, Silvia Fasano, and Gilda Cobellis
Research Article (8 pages), Article ID 2750501, Volume 2020 (2020)

Gestational Exposure to Synthetic Steroid Hormones Impaired Sperm Quantity and Quality in Wistar Rats
Samy Ismail Ahmed, Aamir Magzoub, Mohammed Saeed Zayed Al-Ayed, Gamal Ali Attia, Basel A. Abdel-Wahab, Masood M. Khateeb, Asim M. Abdalla, Heitham M. Mohammed, Aymen N. Eldeen A. Elkareem, and Ali Gadkarim A. Salih
Research Article (6 pages), Article ID 1814867, Volume 2020 (2020)

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 3240198, 15 pages
https://doi.org/10.1155/2020/3240198

Research Article

Quantitative Analysis of the Proteome and the Succinylome in the Thyroid Tissue of High-Fat Diet-Induced Hypothyroxinemia in Rats

Baoxiang Hu,[1,2,3,4] **Meng Zhao**,[1,2,3,5] **Dandan Luo**,[1,2,3] **Chunxiao Yu**,[2,3] **Shanshan Shao**,[2,3] **Lifang Zhao**,[2,3] **Yashuang Yang**,[1,2,3] **Xiaohan Zhang**,[1,2,3] **Jiajun Zhao**,[1,2,3,5] **and Ling Gao**[2,3,5,6]

[1]*Department of Endocrinology, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China*
[2]*Shandong Provincial Key Laboratory of Endocrinology and Lipid Metabolism, Jinan, Shandong, China*
[3]*Institute of Endocrinology and Metabolism, Shandong Academy of Clinical Medicine, Jinan, Shandong, China*
[4]*Department of Cardiology, Zibo Central Hospital, No. 54 Gongqingtuan West Road, Zibo 255036, Shandong, China*
[5]*Department of Scientific Center, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China*
[6]*Scientific Center, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China*

Correspondence should be addressed to Jiajun Zhao; jjzhao@medmail.com.cn and Ling Gao; linggao@sdu.edu.cn

Received 12 March 2020; Revised 14 June 2020; Accepted 22 June 2020; Published 23 July 2020

Guest Editor: Matilde Cescon

Hypothyroidism is a common disease, and its molecular mechanism still needs further investigation. Lysine succinylation is found to be involved in various metabolic processes associated with hypothyroidism. We performed quantitative analysis on lysine succinylome in thyroids of rats with hypothyroxinemia, which was induced through the administration of a high-fat diet. Overall, 129 differentially expressed proteins were quantified. Downregulated proteins were enriched in the thyroid hormone synthesis and thyroid hormone signaling pathways and were mainly localized in the mitochondria. In addition, 172 lysine succinylation sites on 104 proteins were obviously changed. Decreased succinylated proteins were involved in diverse metabolic pathways and were primarily localized in mitochondria. Finally, the mitochondrial oxygen consumption rates of human normal thyroid epithelial cells were measured to further verify the role of lysine succinylation. The mitochondrial oxygen consumption rates were markedly blunted in the cells treated with palmitic acid (all $p < 0.05$), and the changes were reversed when the cells were treated with palmitic acid and desuccinylase inhibitor together (all $p < 0.05$). Thus, we theorize that the thyroid differentially expressed proteins and changed succinylation levels played potential roles in the mitochondria-mediated energy metabolism in the high-fat diet-induced hypothyroxinemia rat model.

1. Introduction

Thyroid hormone, synthesized and secreted by the thyroid gland, plays a crucial role in the normal development, differentiation, and metabolism of human beings [1]. Disturbances in thyroid homeostasis may result in several thyroid disorders such as hypothyroidism. Hypothyroidism is a disorder of the endocrine system that results from low production of thyroid hormone thyroxine (TT4) from the thyroid gland. This leads to metabolic dysfunction because thyroid hormone is an essential regulator of glucose-lipid metabolism and energy homeostasis. Hypothyroidism also leads to a rise in the concentration of thyrotropin (TSH) through the negative feedback of the hypothalamus-pituitary-thyroid axis [2]. Primary hypothyroidism, caused by a dysfunction of the thyroid itself, is the main cause of hypothyroidism [3]. The onset of hypothyroidism in adults is often subtle presenting with a range of nonspecific symptoms. However, severe untreated hypothyroidism may result in poor prognoses, such as heart failure, psychosis, and even coma [3]. So far, possible measures for the treatment of hypothyroidism include improvements in symptoms and

prevention of adverse event. Undoubtedly, hypothyroidism places a huge burden on the economy and greatly lowers the quality of the patient's life. Thus, it is essential to investigate the pathogenesis and explore novel treatment strategies.

Posttranslational modifications (PTMs), which refer to covalent modifications introduced to amino acids of proteins either enzymatically or nonenzymatically, are key mechanisms for increasing proteomic diversity and exert crucial effects on biological function in a variety of species [4–7]. PTMs modulate protein properties through proteolytic cleavage of regulatory subunits, addition of a modified group to one or more amino acids, or degradation of entire proteins, thus determining activity status, localization, turnover, and interactions with other molecules [8].

Lysine, as the most common posttranslation modified amino acid residue, is critical for the formation of protein structures and regulation of protein functions. Lysine residues can be subjected to various PTMs, such as methylation, acetylation, biotinylation, ubiquitination, ubiquitin-like modifications, propionylation, and butyrylation [9–13]. These lysine PTMs play important roles in cellular physiology and pathology, thereby influencing almost all aspects of cell biology and pathogenesis [14–17]. Lysine succinylation is one of significant posttranslational protein modifications, which can occur on cytosolic, nuclear, and mitochondrial proteins by a nonenzymatic chemical reaction [18] and enzymatic catalytic reaction. The former succinylation originates directly from succinyl-CoA, which can be generated from the TCA cycle, lipids, and amino acid metabolism, and the enzymatic succinylation of lysine takes place by lysine succinyltransferase. Lysine succinylation has been identified and verified as an important form of PTM and is involved in a diverse array of cellular functions associated with thyroid diseases [19–21]. Cinzia Puppina et al. have found that acetylated levels of lysine at positions 9–14 of H3 histone (H3K9-K14ac) were significantly higher in follicular adenomas, papillary thyroid carcinomas, follicular thyroid carcinomas, and undifferentiated carcinomas than in normal tissues [22]. Andrea Henze et al. reported that oxidative modifications of Cys10 seemed to affect the binding of T3 to transthyretin and provided a sensitive mechanism for adjusting thyroid hormone availability [23]. The role of lysine succinylation in thyroid diseases is still unknown and needs further investigation.

Our previous studies [24, 25] have found that excess intake of dietary fat induced decreased serum TT_4 and FT_4 concentrations in parallel with elevated serum TSH concentration, as well as abnormal morphology and lipid profile change of the thyroid gland, providing evidence for the correlation between lipid profiles and organ function, as well as a new prospect for understanding the pathogenesis of hypothyroidism. However, the underlying molecular mechanism remains unclear and needs further investigation.

Liquid chromatography-tandem mass spectrometry-(LC-MS/MS-) based proteomic analysis has emerged as a powerful tool for studying disease mechanisms due to its high throughput and accuracy [20, 26, 27]. In the present study, to investigate which PTM played a role in hypothyroxinemia, we detected four types of lysine acylations by western blotting, including succinylation, crotonylation, 2-hydroxybutyrylaion, and malonylation. A significant change in lysine succinylation was observed in the HFD group relative to the control group. Then, we carried out label-free-based quantitative analyses on the global proteome and lysine succinylome of thyroid tissues in the HFD-induced thyroid dysfunction rat model using LC-MS/MS methods. A series of bioinformatics analyses were conducted to explore the underlying molecular mechanisms of hypothyroxinemia and lysine succinylation's involvement. We aimed to explore the association between lysine succinylation and hypothyroidism and to evaluate potential diagnostic biomarkers and therapeutic targets.

2. Materials and Methods

2.1. Experimental Design and Workflow. We compared the protein expression profile and succinylation level in the rat thyroid tissue between a high-fat diet (HFD) study group and a chow-diet (CD) control group. The experiment procedures consisted of four key steps as follows: (1) the establishment and sample collection in a hypothyroxinemia rat model, as previously described [24, 25]; (2) label-free-based quantitative proteomics, including protein extraction, trypsin digestion, high-performance liquid chromatography (HPLC) fractionation, and antibody-based affinity enrichment of lysine succinylated peptides; (3) LC-MS/MS analyses; and (4) bioinformatics analyses. Three biological replicates were performed for the global proteome and succinylome analyses. The procedure is described in detail in the following paragraphs.

2.2. Animal Model and Ethics Statement. Twenty-six male SD rats at 6-week-old (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) weighing 190–210 g were fed in the experimental animal center of Shandong Provincial Hospital, Shandong University. The rats were maintained at a constant temperature and humidity and were rendered a 12 h : 12 h light–darkness cycle. The rats were randomly and equally divided into the CD control group ($n = 13$) and the HFD study group ($n = 13$) (the detailed composition of fatty acids in diets is shown in Supplementary Table S1). The animals were weighed weekly and fed for 24 weeks. At the 24^{th} week of feeding, all rats were fasted for 12 hours before sacrifice. All experiment protocols were approved by the Animal Ethics Committee of Shandong Provincial Hospital, Shandong University.

2.3. Serum Thyroid Function Parameters Analysis. Serum TT_4, FT_4, and TSH concentrations were measured at the end of the experiment. Blood samples were collected by inferior vena cava puncture. Serum TT_4, FT_4, and TSH were measured by using ELISA kits (CUSABIO, Wuhan, China). All procedures were carried out in accordance with the instructions provided by the manufacturers.

2.4. Protein Extraction and Trypsin Digestion. Thyroid tissue samples from 4, 4, and 5 rats were pooled as three biological replicates, respectively. Each sample was grinded by liquid nitrogen into cell powder and then transferred to a 5 mL centrifuge tube. Following the addition of four volumes of lysis buffer (8 M urea, 1% protease inhibitor cocktail, 3 μM TSA, 50 mM NAM, and 2 mM EDTA) into the centrifuge tube, sonication on ice was performed three times. After centrifugation at 12,000 g at 4°C for 10 min, the remaining debris was removed, and the supernatant was collected. Finally, a BCA kit (Beyotime Institute of Biotechnology, Shanghai, China) was utilized to determine the protein concentration according to the manufacturer's instructions.

In preparation for digestion, the protein solution was reduced (5 mM dithiothreitol, 30 min, 56°C) and alkylated (11 mM iodoacetamide, 15 min, room temperature in darkness). The urea concentration was then diluted to less than 2 M by adding 100 mM NH_4HCO_3. Finally, trypsin (Promega Corporation, Fitchburg, Wisconsin, United States) was added at 1 : 50 enzyme-to-substrate mass ratio and incubated overnight for the first digestion, followed by the addition of trypsin at 1 : 100 enzyme-to-substrate mass ratio for an additional 4 h digestion.

2.5. HPLC Fractionation and Antibody-Based Affinity Enrichment. The tryptic peptides were then fractionated into several fractions by high-pH reverse-phase HPLC using Agilent 300 Extend C18 columns (5 μm particles, 4.6 mm ID, 250 mm length). Briefly speaking, peptides were first separated into 60 fractions with a gradient of 8%–32% acetonitrile (ACN, pH 9.0) for over 60 min. Afterwards, the peptide fractions were combined into 4 fractions and dried by vacuum centrifuging.

Enrichment was implemented by immunoprecipitation in accordance with previous studies [28, 29]. Briefly, to enrich lysine-succinylation modified peptides, tryptic peptides were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, and pH 8.0) and then incubated overnight with prewashed antisuccinyl lysine antibody agarose beads (catlog no. PTM402; PTM Bio, Hangzhou, China) at 4°C with gentle shaking. Finally, the bound peptides were eluted from the beads with 0.1% trifluoroacetic acid (TFA), combined, and vacuum-dried. Before LC-MS/MS analysis, the obtained peptides were desalted with C18 ZipTips (Millipore) according to the manufacturer's instructions.

2.6. LC-MS/MS Detection, Database Search, and Quantification Analysis. The tryptic peptides were resuspended in solvent A (0.1% formic acid in 2% ACN) and then directly loaded onto a reversed-phase analytical column (15 cm length, 75 μm ID; PTM bio, Hangzhou, China). A constant flow rate of 700 nl/min was established on an EASY-nLC 1000 UPLC system, with a gradient consisting of 9%–25% solvent B (0.1% formic acid in 90% ACN) for 38 min, 25%–40% for 14 min, climbing to 80% for 4 min, and holding at 80% for the last 4 min.

The peptides were subjected to a nanospray ionization (NSI) source, followed by tandem mass spectrometry (MS/MS) in a Q Exactive™ Plus (Thermo Fisher Scientific) coupled online to an ultraperformance liquid chromatograph (UPLC). Intact peptides and succinylated peptides were detected in the Orbitrap at a resolution of 70,000 and a m/z scan ranging from 350 to 1800. The peptides were then selected using 28% normalized collision energy (NCE) for MS/MS analyses, and the ion fragments were detected using the Orbitrap at a resolution of 17,500. Data-dependent acquisition (DDA) procedures that alternated between one MS scan followed by 15 and 20 MS/MS scans were applied to collect the top 15 and 20 precursor ions of peptides and succinylated peptides above a threshold ion count of 10,000 in the MS survey scan with 30.0 s and 15.0 s dynamic exclusions, respectively. The electrospray voltage applied was 2.1 kV. The automatic gain control (AGC) was utilized to prevent overfilling of the ion trap, and 50,000 ions were accumulated for the generation of MS/MS spectra. The maximum injection time was set as 200 ms and 100 ms for peptides and succinylated peptides, respectively.

The acquired MS/MS data were processed and analyzed with the MaxQuant search engine (v.1.5.2.8). Tandem mass spectra were searched against the UniProt rat database (29,795 sequences) concatenated with protein sequences of common contaminants (such as hemoglobin, keratin, and lactoglobulin) and a reverse decoy database. Trypsin/P was specified as a cleavage enzyme allowing up to two missing cleavages, as well as five modifications per peptide. The mass error was set as 20 ppm in the first search and 5 ppm in the main search for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on cysteine was specified as a fixed modification, whereas acetylation on the protein *N*-terminal and oxidation on methionine were specified as variable modifications. For succinylome analysis, succinylation on lysine was also set as variable modifications. The false discovery rate (FDR) thresholds for the identification of PTM levels, peptides, and proteins were adjusted to 0.01. The minimum length of peptide was set as 7 amino acid residues.

The quantitative values of each sample in three replicates were obtained by LFQ intensity. The first step is to calculate the differential concentration of the protein between the two samples. First, calculate the average value of the quantitative values of each sample in multiple replicates, and then, calculate the ratio of the average values between the two samples. The ratio is used as the final quantitation. For normalization to succinylated peptides, the naked intensities of succinylated peptides were first measured and then were divided by the corresponding protein intensities [30]. To calculate the significant p value of differential concentration between two samples, the relative quantitative values of each sample were taken as log2 transform (so that the data conform to the normal distribution), and p value was calculated by the two-sample two-tailed t-test method. p value < 0.05 and protein ratio >1.5 were regarded as upregulation. p value < 0.05 and protein ratio $<1/1.5$ were regarded as downregulation.

2.7. Bioinformatics Analysis. The raw proteome and succinylome mass spectrometric data have been deposited to the ProteomeXchange (https://www.ebi.ac.uk/pride) with identifier PXD012814. Gene ontology (GO) analyses were derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/) and GO annotation (http://geneontology.org/) to classify all identified proteins into three categories: biological process, cellular component, and molecular function. A cutoff of absolute fold change ≥1.5 was employed to identify the differentially expressed proteins. The functional pathways of all quantified proteins or succinylated proteins were annotated by performing the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (http://www.genome.jp/kegg/). The functional enrichment analyses were carried out to reveal the differentially expressed proteins enriched in all identified proteins and succinylated proteins. When performing bioinformatics analysis, a two-tailed Fisher's exact test was performed, and a corrected p value < 0.05 was considered significant. Protein-protein interactions were analyzed by STRING (http://string-db.org/) using differential proteins and succinylated proteins with significant abundance changes as input. The required confidence score was set as >0.700 for highly confident interactions. The results were visualized using the Cytoscape package.

2.8. Cell Culture and Reagents. The human normal thyroid epithelial cell line Nthy-ori3-1 (ECACC, Wiltshire, UK) was cultured in RPMI-1640 (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; ExCell Bio, Shanghai, China), penicillin (100 IU/ml), streptomycin (100 IU/ml), and L-glutamine (2 mM) at 37°C in a humidified atmosphere containing 5% CO_2.

For the measurement of mitochondrial functions, briefly, palmitic acid (PA) from 50 mM stock solution was warmed and freshly diluted in 2.5 mM BSA-PBS. The diluted PA solution was warmed to clear in a 55°C water bath. Then, the solution of PA and nicotinamide (NAM) was added to the cultures, respectively. Cells were divided into three groups according to different treatments (the NC group, the PA group, and the PA + NAM group).

For immunoprecipitation, cells divided into four groups (the control group, the NAM group, the PA group, and the NAM + PA group). NAM, PA, and NAM + PA groups were treated with NAM, PA, and both NAM and PA, respectively. All reagents were purchased from Sigma-Aldrich (Saint Louis, USA) unless otherwise stated.

2.9. Measurement of Mitochondrial OCR. The measurement of OCR was performed using an XF96 Analyzer (Seahorse Bioscience, USA) according to the manufacturer's instructions. In brief, approximately 7×10^3 cells per well were seeded onto the Seahorse XF96 cell culture microplate (Seahorse Bioscience, USA) and cultured for 24 hours. After the administration of PA (0.2 mM) and NAM (10 mM), the cells were cultured for another 24 hours. Then, the microplate was incubated in the low-buffered and non-bicarbonated assay medium (XF base medium with 2 mM glutamine, 1 mM sodium pyruvate, and 25 mM glucose) in a non-CO_2 incubator at 37°C for 1 hours. Then, OCRs were measured in an XFe 96 extracellular flux analyzer (Seahorse Bioscience) for 3 periods with 3 min of mixing in each cycle. The results were normalized to the corresponding total protein concentration per well.

2.10. Immunoprecipitation. Cells were cultured for 24 hours. After the administration of PA (0.2 mM) and NAM (10 mM), the cells were cultured for another 24 hours. Then, cells were washed three times with ice-cold PBS and lysed in 1 ml ice-cold RIPA lysis buffer (50 mM Tris, 150 nM NaCl, 1% sodium deoxycholate, 1% Triton-X-100, 1 mM EDTA, 0.1% SDS, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 mM NAM). Cells were scraped off from plates, and cell lysates were centrifuged at 12,000 g for 15 minutes. Supernatants were collected, and protein concentration was measured by a BCA kit. 500 μg of total protein was used for IP. Proteins were incubated with the primary antibody overnight at 4°C with gentle rocking. Immunocomplexes were immunoprecipitated using protein A-agarose beads. The immunoprecipitate was washed four times with lysis buffer. Finally, each bead pellet was resuspended in 20 μl of 2× reducing loading buffer (130 mM Tris pH 6.8, 4% SDS, 0.02% bromophenol blue, 20% glycerol, and 100 mM DTT) and boiled at 100°C for 5 min. Samples were stored at −80°C, followed by Western blotting.

2.11. Western Blotting. Equal amounts of protein from different samples were subjected to 8% SDS-PAGE, followed by electrotransfer from the gel to polyvinylidene difluoride membranes (Millipore).The membrane was blocked with 5% (w/v) skim milk in TBST and incubated overnight at 4°C with the pan antisuccinyl lysine antibody (1 : 1000 dilution; PTM Biolabs), anti-sirt5 antibody (1 : 1000 dilution; CST), and anti-GAPDH antibody (1 : 5000; Proteintech, 66009-1-Ig). Following the primary antibodies, the membranes were incubated with the corresponding secondary antibodies at 1 : 5000 dilution for 1 h at room temperature. Immune complexes were detected using an Amersham Imager 600 (General Electric Company). The same membrane was reincubated with anti-GAPDH antibodies. The GAPDH protein was used as a loading control for total proteins.

2.12. Statistical Methods. Quantitative data were presented as the mean ± SEM and were processed using GraphPad Prism 6.0 (La Jolla, CA, USA) and SPSS version 22.0 (Chicago, IL, USA). One-way ANOVA followed by Turkey's post hoc test was performed for multiple comparisons. A p value < 0.05 was considered significant when comparing HFD thyroid samples with their corresponding CD thyroid samples.

3. Results

3.1. HFD-Induced Hypothyroxinemia. To observe the thyroid function, we measured serum TT_4, FT_4, and TSH. As shown in Figure 1, the HFD group exhibited decreased

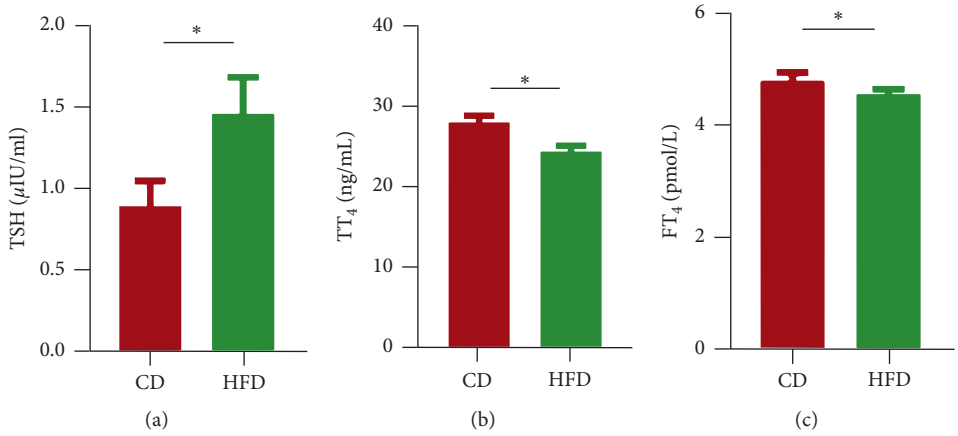


Figure 1: Thyroid hormones measurement. Serum TSH (a), TT_4 (b), and FT_4 (c) were measured using ELISA kits in rats in the chow-diet (CD) control group or the high-fat diet (HFD) study group. Hypothyroxinemia in the HFD group was observed. Error bars represent the mean ± SD. $^*p < 0.01$ versus the CD group.

concentration of TT_4 ($p < 0.01$) and FT_4 ($p < 0.01$) in parallel with elevated concentration of TSH ($p < 0.01$). These results indicate that the establishment of the hypothyroxinemia rat model was successful.

3.2. General Characterization of the Quantitative Proteome in Rat Thyroid Tissues. Label-free-based quantitative proteomics was performed using HPLC fractionation and high-resolution LC-MS/MS analysis. Pairwise Pearson's correlation coefficients displayed sufficient reproducibility of the experiment (Supplementary Figure S1A in the Supplementary Material for comprehensive image analysis). A total of 3869 proteins were identified, among which 2982 proteins were quantitative (Supplementary Table S2). Differentially expressed proteins were filtered with a fold-change threshold >1.5 (p value <0.05) for upregulation and a ratio < 1/1.5 (p value <0.05) for downregulation in the thyroid of the rats with HFD relative to the control. A total of 129 proteins were quantified as differentially expressed proteins between the two groups, including 69 upregulated and 60 downregulated proteins, which is exhibited by volcano plot (Figure 2(a)). Then, these differentially expressed proteins were annotated by performing intensive bioinformatics analyses.

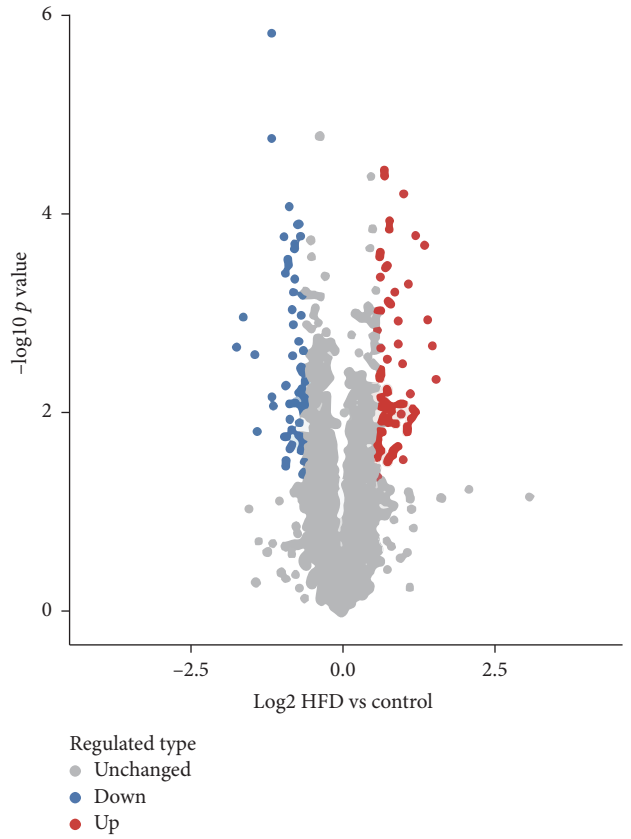
Comparing rat thyroid proteome data with Protein Atlas Publica database, some hallmark proteins in the thyroid tissue can be found in our detected results (Figure 2(b)). For example, thyroglobulin (Tg) is the most abundant among all proteins in MS detected, which acts as a substrate for the synthesis of the thyroid hormones, thyroxine (T4) and triiodothyronine (T3). Thyroid peroxidase is also high level expressed, which is involved in the pathway of thyroid hormone biosynthesis. Other specific proteins such as iodotyrosine deiodinase 1 and calcitonin gene-related peptide 2 in Protein Atlas thyroid database exist in our protein detected table too (Supplementary Table S2). All of these proved our mass spectrum proteomics qualification, and quantification is credible.

GO biological process and molecular function enrichment analysis were performed to all quantified proteins. GO biologic process enrichment analysis shows that the most significant enrichment is metabolic process, including single-organism metabolic process, small molecule metabolic process, and organonitrogen compound metabolic process (Figure 2(c)). For GO molecular function enrichment, poly(A) RNA binding, protein binding, and cadherin binding involved in cell-cell adhesion are top three GO items.

3.3. Enrichment Analysis of the Differentially Expressed Proteins. Enrichment analyses were performed to identify GO terms, KEGG pathways, and domains that were significantly enriched.

GO analyses were conducted to characterize the biological processes and molecular functions of the differentially expressed proteins. As shown in Figures 3(a) and 3(b), among cellular components, the expression of proteins localized to the ribosome and ribosome subunit increased, while the expression of proteins localized to the mitochondrion and ATPase complex significantly decreased. Among the molecular functions, the structural constituents of ribosomes and the structural molecule activity were upregulated, while the ATPase activity was downregulated. In the biological process category, the upregulated proteins were markedly enriched in several metabolic processes (including peptide, cellular amide, and macromolecule metabolism), biosynthetic processes (such as peptide, amide, macromolecule, and organic substance biosynthesis), and translation. In contrast, some downregulated proteins were enriched in a number of metabolic processes including the nitrogen cycle and sulfur metabolism.

The KEGG pathway enrichment analyses were also performed to further investigate the functions of these differentially expressed proteins. Consistent with the results of GO analyses, the results show that the ribosome pathway was the most prominent enriched pathway for upregulated



(a)

Protein accession	Protein description	Gene name	Coverage (%)	MW (kDa)	Score
P06882	Thyroglobulin	Tg	39.3	304.64	323.31
FILN48	Thyroid peroxidase	Tpo	42.8	101.48	323.31
PI4650	Thyroid peroxidase	Tpo	40.8	101.46	N/A
Q5BK17	Lodotyrosine deiodinase 1	Iyd	23.5	32.846	37.923
G3V7G7	Calcitonin gene-related peptide 2	Calcb	28.4	14.896	67.434
A0AOG2JXM4	Lodothyronine deiodinase	Diol	34	29.58	27.352
FILR66	Sodium/iodide cotransporter	Slc5a5	8.3	65.228	9.8377
D3Z8P6	Anion exchange transporter	Slc26a7	2.8	65.939	2.9963

(b)

Figure 2: Continued.

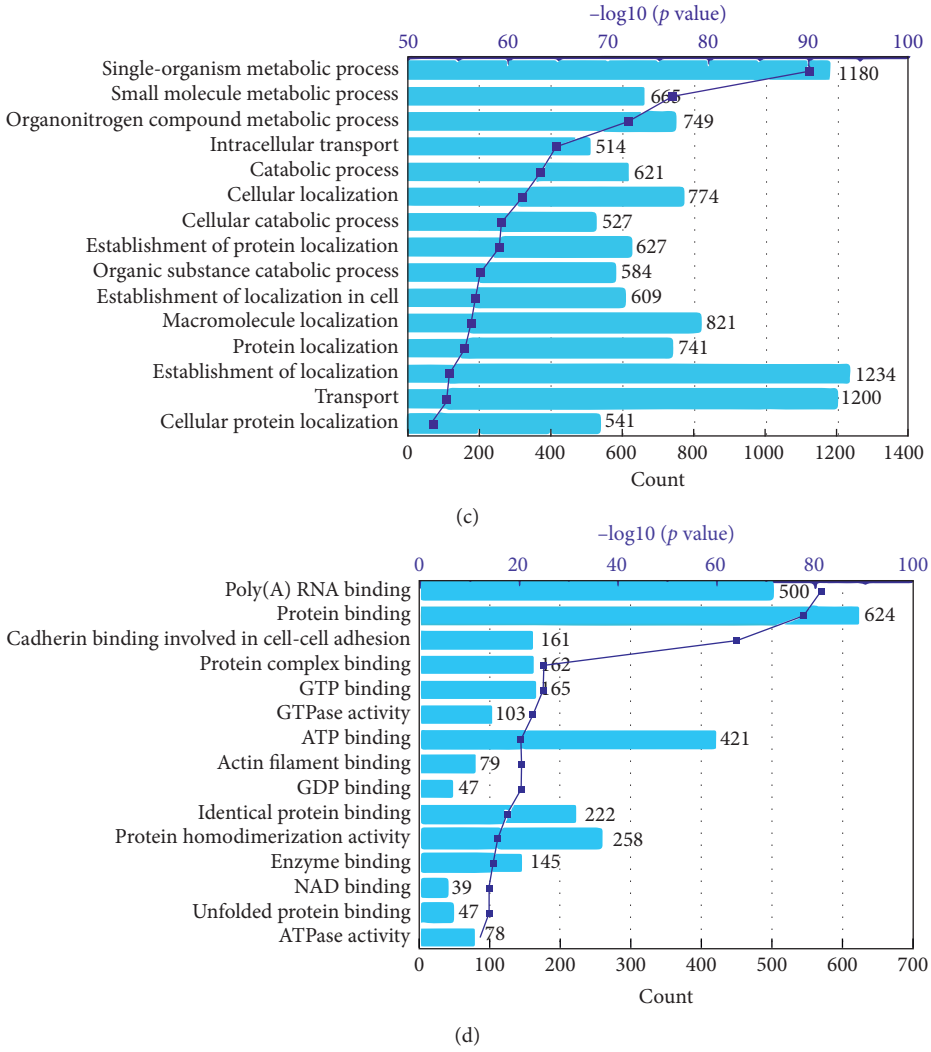


FIGURE 2: General characterization of the quantitative proteome. (a) Volcano plot illustrating significantly differential abundant proteins in proteome analysis. The –log10 (p value) is plotted against the log2 (ratio HFD/control). (b) Table illustrating the specific proteins in thyroid detected. (c, d) GO biologic process and molecular function enrichment analysis, respectively; bars length represents genes number and dots indicate –log10 (p value) corresponding GO items.

proteins (Figure 3(c)). Meanwhile, downregulated proteins were observed to be enriched in the thyroid hormone signaling pathway and thyroid hormone synthesis, indicating that these pathways may play essential roles in the development of hypothyroxinemia (Figure 3(d)).

3.4. General Characterization of Quantitative Succinylome in Rat Thyroid Tissues. Label-free-based quantitative lysine succinylome analysis was performed using antibody-based affinity enrichment, followed by LC-MS/MS analysis. Altogether, 685 succinylation sites in 250 proteins were identified, among which 621 succinylation sites on 229 proteins were quantified and normalized to the proteome data (Supplementary Table S3). With a quantification ratio of >1.5 (p value < 0.05) as the upregulation threshold and <1/1.5 (p value < 0.05) as the downregulation threshold, 172 succinylation sites corresponding to 104 proteins showed different succinylation levels in three repeated experiments (7 upregulated succinylated sites on 5 proteins, 165 downregulated succinylated sites on 99 proteins, and the HFD group compared with the CD group), which is exhibited by volcano plot (Figure 4(a)). The average peptides mass error was <10 ppm, indicating a high mass accuracy of the MS data (Supplementary Figure S1B in the Supplementary Material for comprehensive image analysis). The lengths of the most identified peptides were 8–20 amino acid residues (Supplementary Figure S1C in the Supplementary Material for comprehensive image analysis).

Compared with the CD group, most lysine succinylation on different proteins undergo downregulated change in the HFD group, moreover, these succinylated proteins are located in mitochondria, including ATP synthase complex and isocitrate dehydrogenase (IDH2). However, several lysine sites succinylation are upregulated on thyroglobulin (Figure 4(b)). GO biologic process enrichment analysis

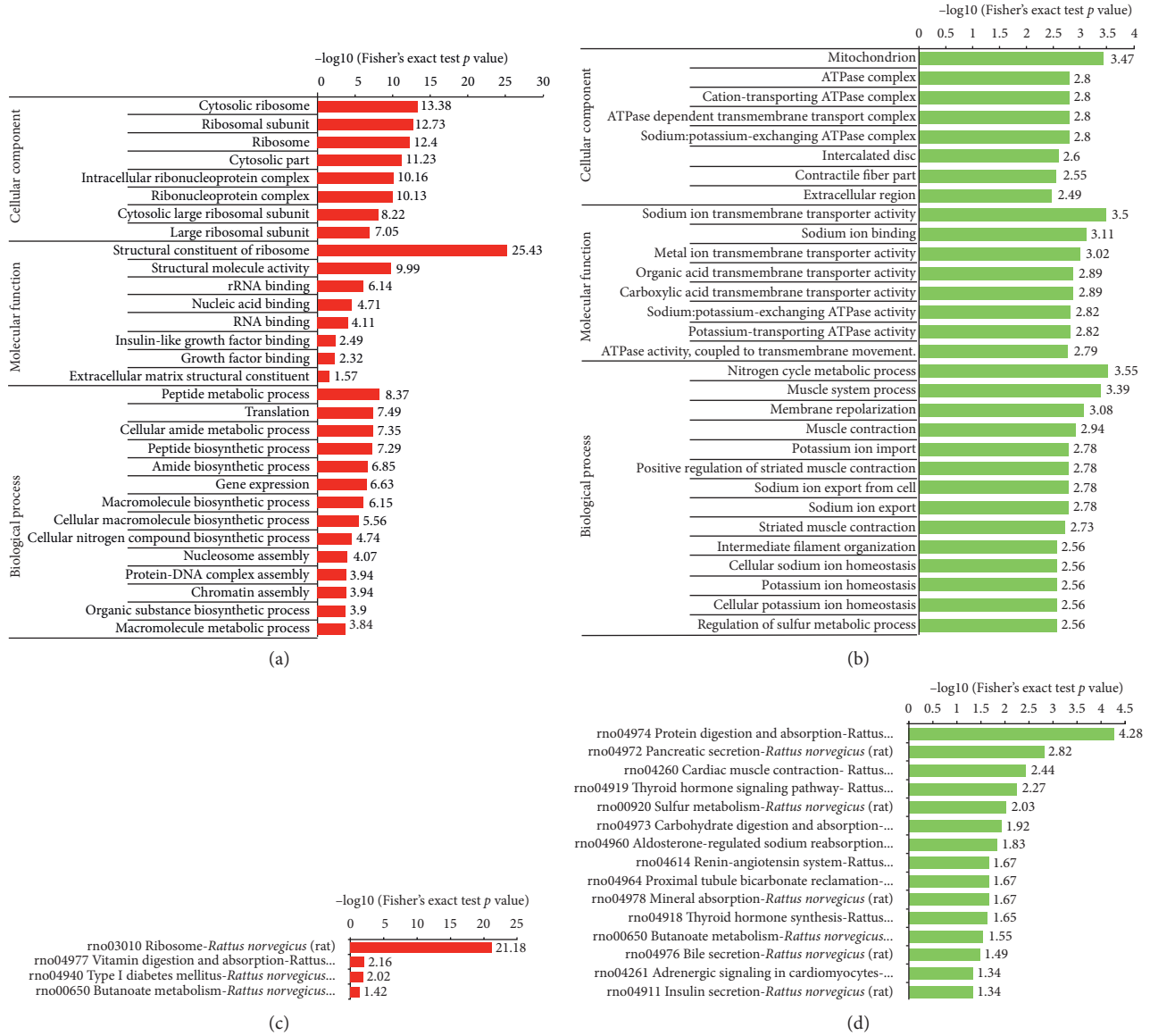
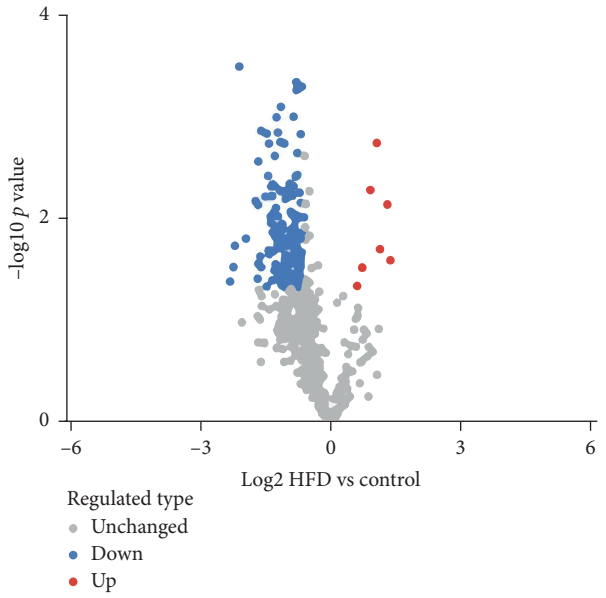


Figure 3: Enrichment analyses of the differentially expressed proteins: (a) upregulated and (b) downregulated proteins were examined by the GO functional enrichment; (c) upregulated and (d) downregulated proteins were examined by the KEGG pathway analysis. Upregulated and downregulated proteins were defined as >1.5 folds and <1/1.5 compared to the control, respectively.

reveals that the tricarboxylic acid cycle, fatty acid beta-oxidation using acyl-CoA dehydrogenase, oxidation-reduction process, fatty acid beta-oxidation, and lipid homeostasis are top five significant GO items (Figure 4(c)). And, molecular function enrichment analysis (Figure 4(d)) shows that fatty-acyl-CoA binding and related metabolism are enriched significantly.

3.5. Enrichment Analysis of the Differentially Changed Succinylated Proteins. As shown in Figures 5(a) and 5(b), among cellular components, the downregulated succinylated proteins mainly exhibited the tricarboxylic acid cycle (TCA cycle) enzyme complex. The most enriched molecular function was the ligase activity. In the biological process category, proteins related to the system process that responded to the lipids were enriched among the upregulated succinylated proteins, while proteins related to the TCA metabolic process, cellular respiration, TCA cycle, citrate metabolic process, energy derivation by oxidation of organic compounds, and aerobic respiration were enriched among the downregulated succinylated proteins.

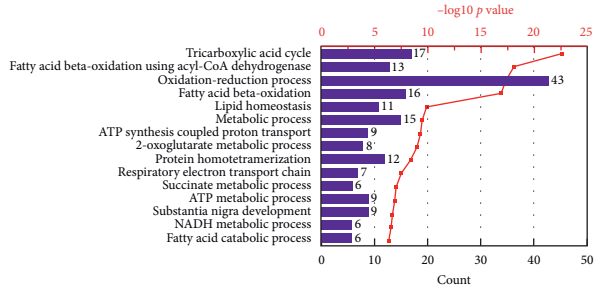
The KEGG pathway enrichment analysis indicated that the citrate cycle (TCA cycle) was the most enriched pathway among the downregulated succinylated proteins. In addition, propanoate metabolism and pyruvate metabolism related pathways were also enriched (Figure 5(c)). Protein domain analysis revealed that the



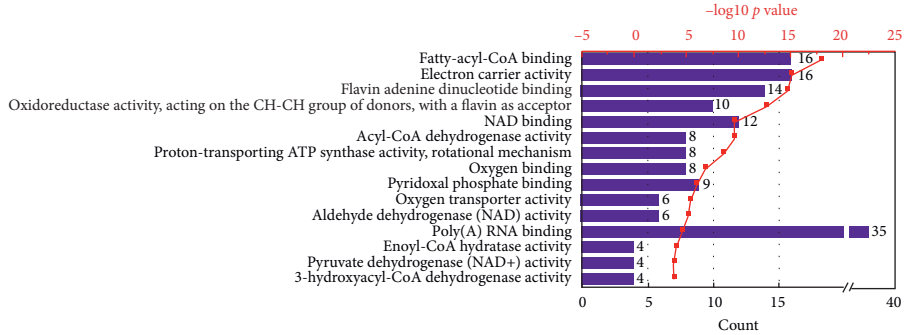
(a)

Gene name	Protein description	Modified sequence	Position	Regulated type	HFD/CD ratio	p value
Idh2	Isocitrate dehydrogenase [NADP] mitochondrial	HYK(1)TDFDK	275	Down	0.586	$5.42E-04$
		EK(1)LILPHVDVQLK	69	Down	0.544	$5.80E-03$
		HAHGDQYK(1)ATDFVVDR	180	Down	0.388	$9.68E-03$
		VEK(1)PVVEMDGDEMTR	48	Down	0.402	$1.38E-02$
		K(1)WPLYLSTK	243	Down	0.587	$1.53E-02$
		GK(1)LDGNQDLIR	384	Down	0.465	$1.57E-02$
		NTIMK(1)AYDGR	256	Down	0.515	$1.58E-02$
Tg	Thyroglobulin	AGEIPACPGPCEEVK(1)FR	930	Up	2.227	$2.04E-02$
		FANLIQSGK(1)FQLHLDSK	1407	Up	2.624	$2.60E-02$
		EAFSEK(1)FLR	986	Up	1.669	$3.07E-02$

(b)



(c)



(d)

Figure 4: General characterization of the quantitative succinylated proteins. (a) Volcano plot illustrating significantly differential abundant succinylation sites analysis. The −log10 (p value) is plotted against the log2 (ratio HFD/control). The −log10 (p value) is plotted against the log2 (ratio HFD/control). (b) Table illustrating significant differential upregulated or downregulated succinylation site corresponding peptides. (c, d) GO biologic process and molecular function enrichment analysis, respectively; bars length represents genes number and dots indicate −log10 (p value) corresponding GO items.

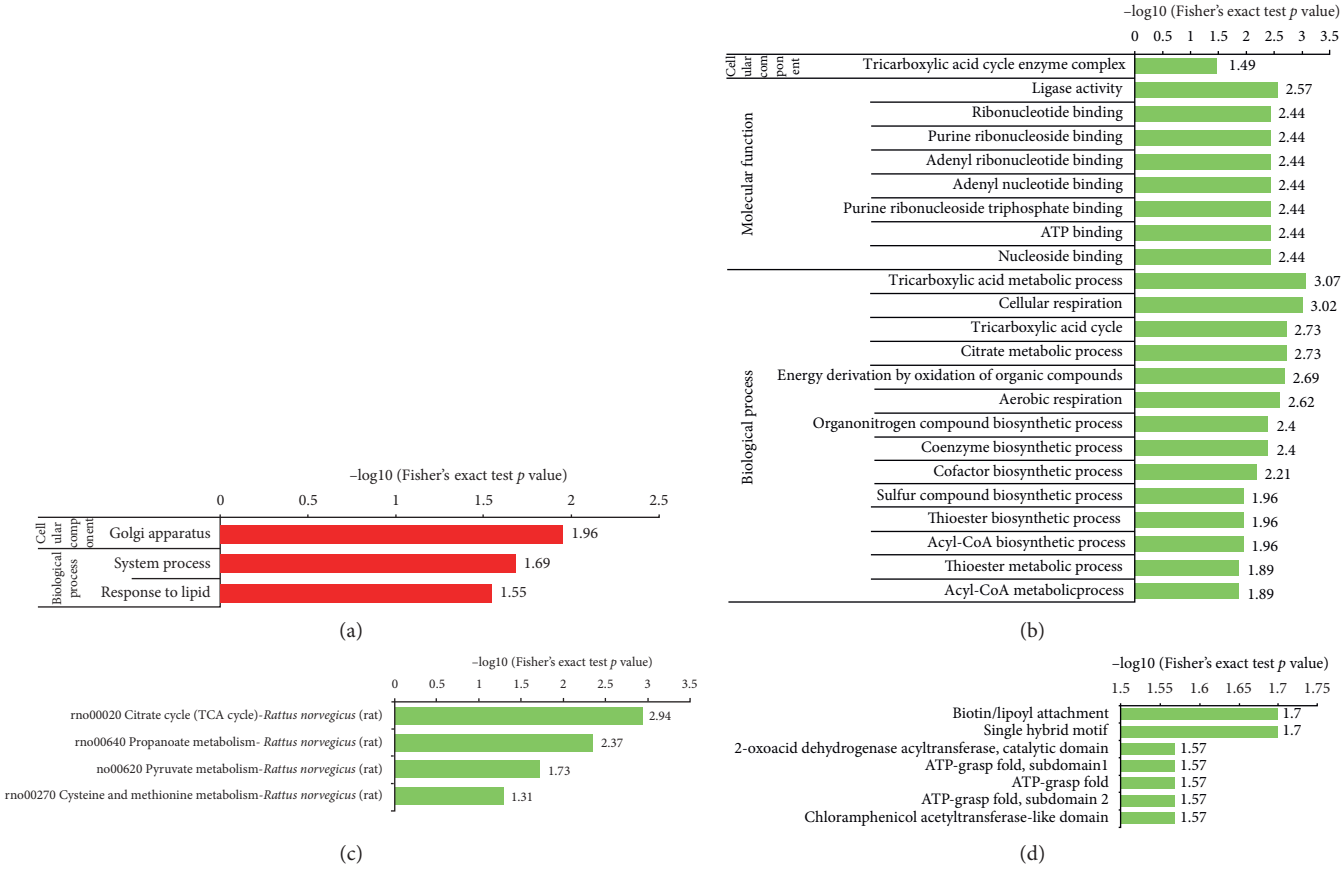


Figure 5: Enrichment analyses of the differentially changed succinylated proteins: (a) upregulated and (b) downregulated succinylated proteins were classified by the GO functional enrichment; downregulated succinylated proteins were examined by (c) KEGG pathway analysis and (d) domain enrichment analysis.

top two significantly enriched terms were biotin/lipoyl attachment and single hybrid motif (Figure 5(d)).

3.6. Differential Proteins and Succinylated Proteins PPI Analysis. To better understand the function of lysine succinylation and hypothyroidism pathology, the differential proteins and succinylated proteins were subjected to a protein-protein interaction (PPI) network analysis using the STRING database (Vision 11.0). STRING defines a metric called "confidence score" to define interaction confidence; we fetched all interactions that had a confidence score ≥0.7 (high confidence). A network of protein-protein interactions was generated and clustered with the Markov cluster (MCL) algorithm [31], which was then visualized using the Cytoscape program (Vision 3.7).

Differential protein-protein net analysis reveals that ribosome proteins interaction is highly clustered, and the proteins of this PPI net cluster is characterized with upregulated coexpression (Figure 6(a)). The differential succinylated proteins interacting net (Figure 6(b)) shows three function clusters: the citrate cycle (TCA cycle), ATP synthase complex, and valine, leucine, and isoleucine degradation, and all of them are downregulated succinylation.

3.7. Measurement of Mitochondrial OCR. To further investigate the role of fatty acids in mitochondrial function and to avoid cofounding factors *in vivo*, OCR representing levels of mitochondrial function were measured in normal human thyroid epithelial cells. As shown in Figure 7, mitochondrial OCRs related to basal respiration, ATP production, and maximal respiration are markedly blunted by palmitic acid exposure (all $p < 0.05$), and the changes were reversed when the cells were treated with palmitic acid and desuccinylase inhibitor together (all $p < 0.05$).

3.8. Verification of Protein Succinylation Levels. To determine the variation of protein succinylation in cell line, we performed protein immunoprecipitation assays coupled with Western blotting to detect isocitrate dehydrogenase 2 (IDH2) succinylation levels. IDH2 was known as a critical enzyme in the tricarboxylic acid cycle. Nthy-ori3-1 cells were treated with NAM or PA or both NAM and PA, respectively. The result showed that IDH2 was indeed succinylated, and its succinylation was inhibited by PA treatment. Although IDH2 succinylation did not show obvious change in response to NAM, both PA and NAM treatment made IDH2 succinylation level recovered compared with PA treatment (Figure 8).

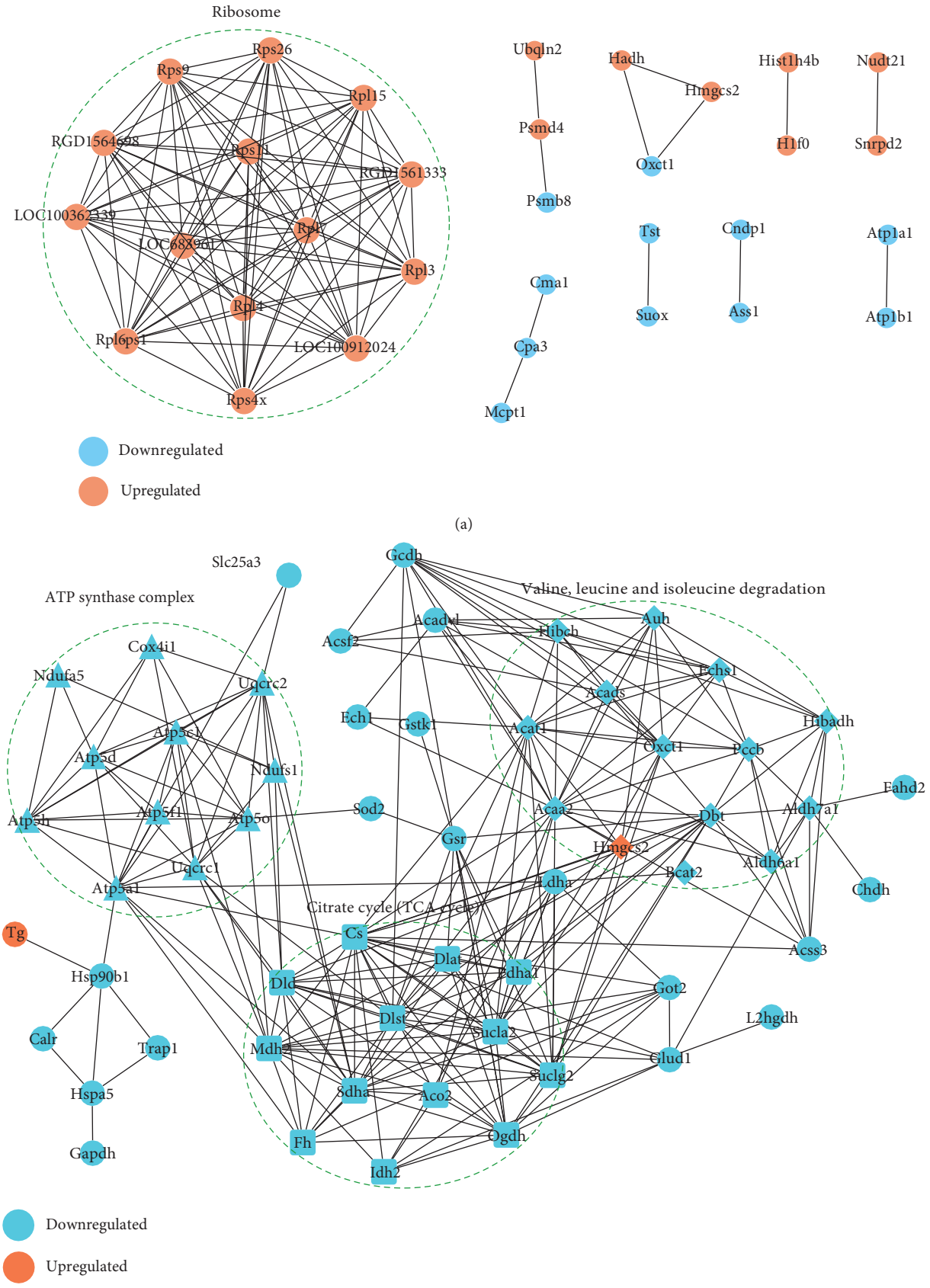


Figure 6: Differential proteins and succinylated proteins interacting net analysis. (a) Differential proteins PPI net. (b) Differential succinylated proteins PPI net.

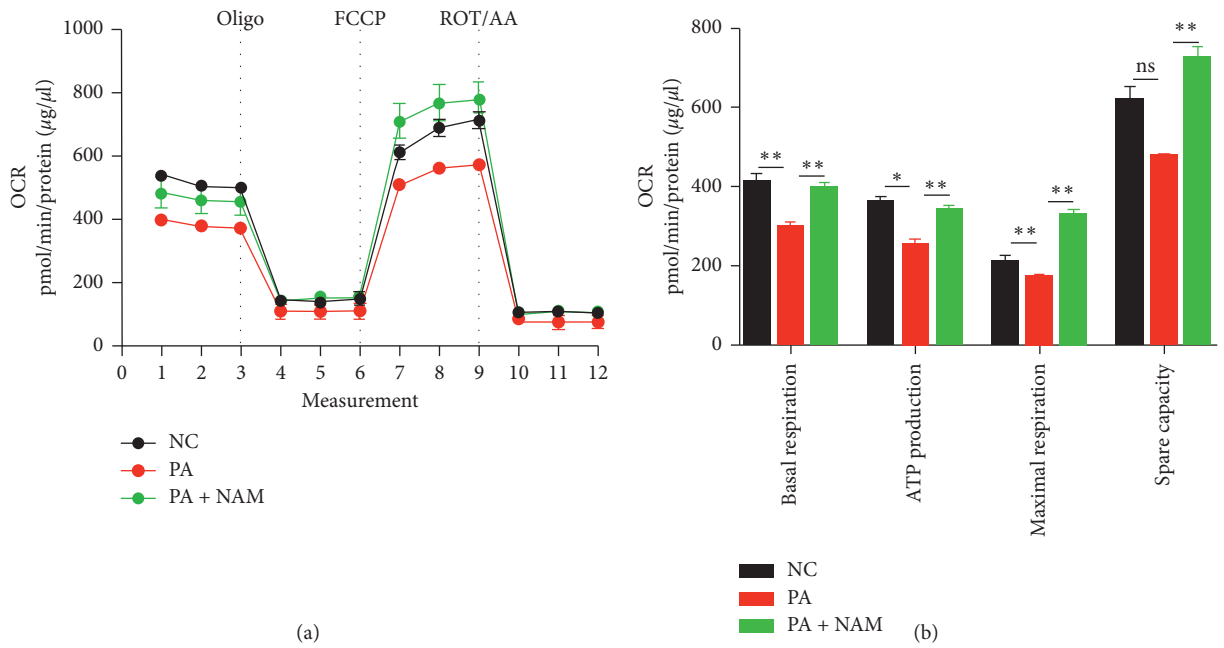


Figure 7: Human normal thyroid epithelial cells were isolated and cultured. The cells were treated with and without palmitic acid (PA) in the presence and absence of a desuccinylation inhibitor (NAM) for 24 hours, respectively. Mitochondrial OCRs were measured using the Seahorse XF96 analyzer (mean ± SEM, $n = 7–8$). OCRs related to the mitochondrial basal respiration, ATP production, maximal respiration, and spare capacity were analyzed and normalized to the corresponding total protein concentration per well, respectively, (ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$). Each datum was obtained from independent three days. One-way ANOVA followed by Turkey's post hoc test was performed for multiple comparisons.

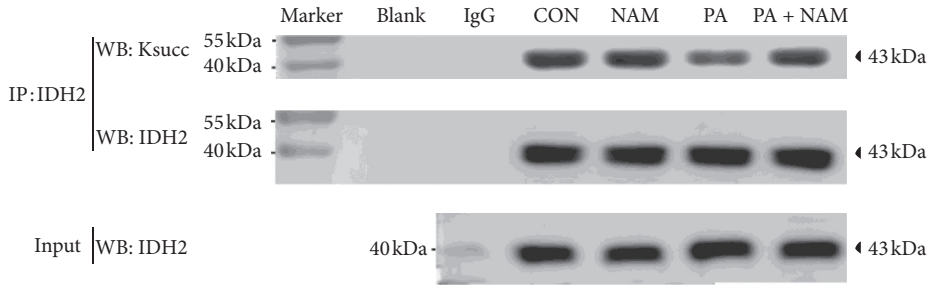


Figure 8: Human normal thyroid epithelial cells were isolated and cultured. The cells were treated with and without palmitic acid (PA) in the presence and absence of a desuccinylation inhibitor (NAM) for 24 hours, respectively. Total proteins of each group were subjected to immunoprecipitation (IP) with an antibody of isocitrate dehydrogenase 2 (IDH2). Succinylation status and expression of IDH2 were evaluated by Western blotting analysis. IDH2 expression showed no obvious change. IDH2 succinylation was inhibited by PA treatment and showed no obvious change in response to NAM alone. After treatment of both PA and NAM, IDH2 succinylation level was recovered compared with PA treatment.

4. Discussion

In present study, a HFD-induced hypothyroxinemia rat model was constructed according to our previous studies [24, 25]. Excess intake of dietary fat induced significant thyroid dysfunction and hypothyroxinemia in rats by decreasing the expression of thyroid hormone synthesis-related proteins, providing evidence for the correlation between lipid profiles and organ function. Then, we employed a quantitative proteomics strategy and LC-MS/MS-based enrichment to investigate global protein and succinylation profiles in thyroid tissues. We identified 129 differentially expressed proteins and 172 differentially expressed succinylation sites, among which several proteins and succinylation sites were localized in the mitochondria and associated with mitochondrial function. To confirm the alterations in mitochondrial respiratory activities, OCR was further employed to verify the LC-MS/MS results.

The proteome data demonstrated changes in the metabolic processes in the HFD-induced hypothyroxinemia rat model. Benard et al. presented a proteome-wide study in HFD-fed mice and detected that fifty-four percent of those differentially expressed proteins were involved in metabolic processes [32]. In addition, Yang et al. revealed that the

metabolic pathways of differentially expressed proteins were possibly related to the HFD-induced decline in male rats' fertility [33]. Furthermore, Tu et al. found that the protein expression levels of intracranial and extracranial atherosclerosis in HFD-fed rabbits were different, which facilitated the diagnosis and treatment of cerebral arteriosclerosis [34]. These proteomics studies provided molecular understandings of HFD-induced pathology and identified potential targets for the development of therapeutics for metabolic syndromes. Thus, we theorize that HFD may play an important role in the formation of hypothyroidism [35, 36].

Recent studies have focused on protein PTMs to identify the potential mechanisms of several diseases. Various types of PTMs such as phosphorylation, lysine acetylation, ubiquitination, propionylation, crotonylation, and succinylation have been discovered with the development of mass spectrometry technology [7, 12, 37]. Particularly, the role of PTMs in regulating cellular energy metabolism has been demonstrated. To investigate which PTM played a role in hypothyroxinemia, we detected four types of lysine acylations by western blotting, including succinylation, crotonylation, 2-hydroxybutyrylaion, and malonylation. A significant change in lysine succinylation was observed in the HFD group relative to the control group. Lysine succinylation, as a newly identified PTM in proteins, is widespread in diverse organisms and impacts various metabolic pathways [38–41]. Thus, we investigated the quantitative protein succinylome in the HFD-induced hypothyroxinemia rat model, with the goal of exploring the possible role of lysine succinylation in HFD-induced hypothyroxinemia.

At the succinylome level, our data indicated a close relationship between lysine succinylation and mitochondria-mediated metabolic regulation. As we all know, mitochondria participate in the metabolism of amino acids, lipids, cholesterol, steroids, and nucleotides. Perhaps, most importantly, mitochondria play a fundamental role in cellular energy metabolism (including the fatty acid β-oxidation and the respiratory chain), which is essential to diverse cellular functions and developmental processes [35, 36]. Recent protein succinylome studies in human renal cell carcinoma tissues have shown that the glycolysis pathway might be regulated through lysine succinylation and play a potential role in renal cell carcinoma progression [42]. Additionally, Song et al. identified that the TCA cycle and pentose phosphate pathway were potential mechanisms of the energy metabolism in human gastric cancer, which might be regulated through lysine succinylation in their core enzymes [43]. Furthermore, it is well known that thyroid hormone participates in energy regulation and metabolic processes, and the loss of thyroid hormone homeostasis is highly associated with various thyroid dysfunctions including hypothyroidism and hyperthyroidism [44, 45]. Thus, lysine succinylation may play a vital role in mitochondrial function and energy metabolism in the HFD-induced hypothyroxinemia rat model [38–41].

Fatty acids, as the major components of triglycerides, were found in increased contents in thyroid tissues of the HFD-induced rat model in previous studies. To further investigate the role of fatty acids in the mitochondrial function and to avoid cofounding factors *in vivo*, fatty acids were used to treat normal human thyroid epithelial cells, thus decreasing the succinylation level. Mitochondrial functions were then investigated *in vitro* in the present study. Palmitic acid, as the most common saturated fatty acid in animals, plants, and microorganisms, was applied. Nicotinamide (NAM), as an inhibitor of SIRT5, which has been reported to catalyze the removal of succinylation [46–48], was then treated to palmitic acid-stimulated cells to inhibit the desuccinylase activity of sirtuins. Mitochondrial OCR was then used to examine the mitochondrial function of normal human thyroid epithelial cells with altered succinylation levels. The results *in vitro* were in accordance with the results *in vivo*, further suggesting that fatty acids might directly influence energy metabolism and play an important role in the formation of hypothyroidism.

To the best of our knowledge, the present study is the first to investigate the succinylome in an HFD-induced hypothyroxinemia rat model. Succinylation level shows significant downregulation in many important proteins, mainly localized in mitochondria. We suppose that the significant succinylation downregulated mitochondrial ATP synthase complex, and mitochondrial IDH2 protein is more likely to accompany with the depressed tricarboxylic acid cycle activity because succinylation depends on intracellular succinyl-CoA levels, and succinyl-CoA can be generated from the TCA cycle, lipids, and amino acid metabolism, for succinylation can occur by a nonenzymatic chemical reaction.

5. Conclusions

This research reveals significant downregulated lysine succinylated proteins mainly localized in mitochondria and cooccur with the depressed mitochondria activity in the HFD-induced hypothyroxinemia rat model. These results expand our understanding of the underlying mechanism of hypothyroidism progression and provide new avenues of exploration with regard to potential treatment strategies for hypothyroidism.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The abstract of this paper was presented at the ENDO 2019 Abstracts-101st Annual Meeting of the Endocrine Society.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors thank the ENDO 2019 Abstracts-101st Annual Meeting of the Endocrine Society for publishing the abstract. The authors thank the Jingjie PTM BioLab (Hangzhou) for

technical assistance in experiments. This research was supported by the National Natural Science Foundation of China (81430020, 81230018, 81600604, 81300644, and 81670720) and the Shandong Science and Technology Commission of China (ZR2016HB07).

Supplementary Materials

Supplementary Table S1: fatty acids composition of diets. Supplementary Figure S1: Pearson's correlation coefficient and quality control of validation of MS/MS data. Supplementary Table S2: all annotated proteins identified and differentially expressed proteins in quantitative proteome of rat thyroid tissues; S2: HFD group and S1: CD group, each group of three biological repetitions. Supplementary Table S3: all annotated succinylated proteins and significantly differential lysine succinylation in quantitative succinylome of rat thyroid tissues. S2: HFD group and S1: CD group, each group of three biological repetitions. (*Supplementary Materials*)

References

[1] W. E. Winter and M. R. Signorino, "Review: molecular thyroidology," *Annals of Clinical and Laboratory Science*, vol. 31, no. 3, pp. 221–244, 2001.

[2] R. S. Lindsay and A. D. Toft, "Hypothyroidism," *The Lancet*, vol. 349, no. 9049, pp. 413–417, 1997.

[3] C. G. Roberts and P. W. Ladenson, "Hypothyroidism," *The Lancet*, vol. 363, no. 9411, pp. 793–803, 2004.

[4] J. A. Cain, N. Solis, and S. J. Cordwell, "Beyond gene expression: the impact of protein posttranslational modifications in bacteria," *Journal of Proteomics*, vol. 97, pp. 265–286, 2014.

[5] A. Hofer and T. Wenz, "Posttranslational modification of mitochondria as a novel mode of regulation," *Experimental Gerontology*, vol. 56, pp. 202–220, 2014.

[6] E. S. Witze, W. M. Old, K. A. Resing, and N. G. Ahn, "Mapping protein posttranslational modifications with mass spectrometry," *Nature Methods*, vol. 4, no. 10, pp. 798–806, 2007.

[7] C. T. Walsh, S. Garneau-Tsodikova, and G. J. Gatto Jr., "Protein posttranslational modifications: the chemistry of proteome diversifications," *Angewandte Chemie International Edition*, vol. 44, no. 45, pp. 7342–7372, 2005.

[8] M. Mann and O. N. Jensen, "Proteomic analysis of post-translational modifications," *Nature Biotechnology*, vol. 21, no. 3, pp. 255–261, 2003.

[9] S. L. Berger, "The complex language of chromatin regulation during transcription," *Nature*, vol. 447, no. 7143, pp. 407–412, 2007.

[10] Y. Chen, R. Sprung, Y. Tang et al., "Lysine propionylation and butyrylation are novel post-translational modifications in histones," *Molecular & Cellular Proteomics*, vol. 6, no. 5, pp. 812–819, 2007.

[11] C. Peng, Z. Lu, Z. Xie et al., "The first identification of lysine malonylation substrates and its regulatory enzyme," *Molecular & Cellular Proteomics*, vol. 10, no. 12, Article ID M111.012658, 2011.

[12] M. Tan, H. Luo, S. Lee et al., "Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification," *Cell*, vol. 146, no. 6, pp. 1016–1028, 2011.

[13] K. Zhang, Y. Chen, Z. Zhang, and Y. Zhao, "Identification and verification of lysine propionylation and butyrylation in yeast core histones using PTMap software," *Journal of Proteome Research*, vol. 8, no. 2, pp. 900–906, 2009.

[14] S. B. Hake, A. Xiao, and C. D. Allis, "Linking the epigenetic 'language' of covalent histone modifications to cancer," *British Journal of Cancer*, vol. 96, pp. R31–R39, 2007.

[15] C. Martin and Y. Zhang, "The diverse functions of histone lysine methylation," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 11, pp. 838–849, 2005.

[16] J.-P. Kruse and W. Gu, "Modes of p53 regulation," *Cell*, vol. 137, no. 4, pp. 609–622, 2009.

[17] A. Hershko and A. Ciechanover, "The ubiquitin system," *Annual Review of Biochemistry*, vol. 67, no. 1, pp. 425–479, 1998.

[18] G. R. Wagner and R. M. Payne, "Widespread and enzyme-independent N^{ϵ}-acetylation and N^{ϵ}-succinylation of proteins in the chemical conditions of the mitochondrial matrix," *Journal of Biological Chemistry*, vol. 288, no. 40, pp. 29036–29045, 2013.

[19] Z. Zhang, M. Tan, Z. Xie, L. Dai, Y. Chen, and Y. Zhao, "Identification of lysine succinylation as a new posttranslational modification," *Nature Chemical Biology*, vol. 7, no. 1, pp. 58–63, 2011.

[20] X. Li, X. Hu, Y. Wan et al., "Systematic identification of the lysine succinylation in the Protozoan parasite *Toxoplasma gondii*," *Journal of Proteome Research*, vol. 13, no. 12, pp. 6087–6095, 2014.

[21] M. Celano, C. Mio, M. Sponziello et al., "Targeting post-translational histone modifications for the treatment of non-medullary thyroid cancer," *Molecular and Cellular Endocrinology*, vol. 469, pp. 38–47, 2018.

[22] C. Puppin, N. Passon, E. Lavarone et al., "Levels of histone acetylation in thyroid tumors," *Biochemical and Biophysical Research Communications*, vol. 411, no. 4, pp. 679–683, 2011.

[23] A. Henze, T. Homann, M. Serteser et al., "Posttranslational modifications of transthyretin affect the triiodonine-binding potential," *Journal of Cellular and Molecular Medicine*, vol. 19, no. 2, pp. 359–370, 2015.

[24] S.-s. Shao, Y.-f. Zhao, Y.-f. Song et al., "Dietary high-fat lard intake induces thyroid dysfunction and abnormal morphology in rats," *Acta Pharmacologica Sinica*, vol. 35, no. 11, pp. 1411–1420, 2014.

[25] X. Zhang, W. Chen, S. Shao et al., "A high-fat diet rich in saturated and mono-unsaturated fatty acids induces disturbance of thyroid lipid profile and hypothyroxinemia in male rats," *Molecular Nutrition & Food Research*, vol. 62, no. 6, Article ID e1700599, 2018.

[26] B. Zhang, J. Wang, J. Wang et al., "Proteogenomic characterization of human colon and rectal cancer," *Nature*, vol. 513, no. 7518, pp. 382–387, 2014.

[27] Q.-S. Yang, J.-H. Wu, C.-Y. Li et al., "Quantitative proteomic analysis reveals that antioxidation mechanisms contribute to cold tolerance in plantain (*Musa paradisiaca* L.; ABB group) seedlings," *Molecular & Cellular Proteomics*, vol. 11, no. 12, pp. 1853–1869, 2012.

[28] L. Xie, W. Liu, Q. Li et al., "First succinyl-proteome profiling of extensively drug-resistant *Mycobacterium tuberculosis* revealed involvement of succinylation in cellular physiology," *Journal of Proteome Research*, vol. 14, no. 1, pp. 107–119, 2015.

[29] Y. Cheng, T. Hou, J. Ping, G. Chen, and J. Chen, "Quantitative succinylome analysis in the liver of nonalcoholic fatty liver disease rat model," *Proteome Science*, vol. 14, no. 1, p. 3, 2016.

[30] Q. Wu, W. Xu, L. Cao et al., "SAHA treatment reveals the link between histone lysine acetylation and proteome in nonsmall cell lung cancer A549 Cells," *Journal of Proteome Research*, vol. 12, no. 9, pp. 4064–4073, 2013.

[31] A. J. Enright, S. Van Dongen, and C. A. Ouzounis, "An efficient algorithm for large-scale detection of protein families," *Nucleic Acids Research*, vol. 30, no. 7, pp. 1575–1584, 2002.

[32] O. Benard, J. Lim, P. Apontes, X. Jing, R. H. Angeletti, and Y. Chi, "Impact of high-fat diet on the proteome of mouse liver," *The Journal of Nutritional Biochemistry*, vol. 31, pp. 10–19, 2016.

[33] X.-Y. Yang, Y.-j. Gu, T. An et al., "Proteomics analysis of testis of rats fed a high-fat diet," *Cellular Physiology and Biochemistry*, vol. 47, no. 1, pp. 378–389, 2018.

[34] Z.-L. Tu, B. Yu, D.-Y. Huang et al., "Proteomic analysis and comparison of intra- and extracranial cerebral atherosclerosis responses to hyperlipidemia in rabbits," *Molecular Medicine Reports*, vol. 16, no. 3, pp. 2347–2354, 2017.

[35] P. F. Chinnery and E. A. Schon, "Mitochondria," *Journal of Neurology, Neurosurgery & Psychiatry*, vol. 74, no. 9, pp. 1188–1199, 2003.

[36] J. Rahman and S. Rahman, "Mitochondrial medicine in the omics era," *The Lancet*, vol. 391, no. 10139, pp. 2560–2574, 2018.

[37] P. Gut and E. Verdin, "The nexus of chromatin regulation and intermediary metabolism," *Nature*, vol. 502, no. 7472, pp. 489–498, 2013.

[38] J. Park, Y. Chen, D. X. Tishkoff et al., "SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways," *Molecular Cell*, vol. 50, no. 6, pp. 919–930, 2013.

[39] M. J. Rardin, W. He, Y. Nishida et al., "SIRT5 regulates the mitochondrial lysine succinylome and metabolic networks," *Cell Metabolism*, vol. 18, no. 6, pp. 920–933, 2013.

[40] F. Wang, K. Wang, W. Xu et al., "SIRT5 desuccinylates and activates pyruvate kinase M2 to block macrophage IL-1β production and to prevent DSS-induced colitis in mice," *Cell Reports*, vol. 19, no. 11, pp. 2331–2344, 2017.

[41] B. T. Weinert, C. Schölz, S. A. Wagner et al., "Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation," *Cell Reports*, vol. 4, no. 4, pp. 842–851, 2013.

[42] N. Zhang, R. Gao, J. Yang et al., "Quantitative global proteome and lysine succinylome analyses reveal the effects of energy metabolism in renal cell carcinoma," *Proteomics*, vol. 18, no. 19, Article ID e1800001, 2018.

[43] Y. Song, J. Wang, Z. Cheng et al., "Quantitative global proteome and lysine succinylome analyses provide insights into metabolic regulation and lymph node metastasis in gastric cancer," *Scientific Reports*, vol. 7, Article ID 42053, 2017.

[44] G. A. Brent, "Mechanisms of thyroid hormone action," *Journal of Clinical Investigation*, vol. 122, no. 9, pp. 3035–3043, 2012.

[45] A. Warner and J. Mittag, "Thyroid hormone and the central control of homeostasis," *Journal of Molecular Endocrinology*, vol. 49, no. 1, pp. R29–R35, 2012.

[46] J. L. Avalos, K. M. Bever, and C. Wolberger, "Mechanism of sirtuin inhibition by nicotinamide: altering the NAD+ cosubstrate specificity of a Sir2 enzyme," *Molecular Cell*, vol. 17, no. 6, pp. 855–868, 2005.

[47] L. Li, L. Shi, S. Yang et al., "SIRT7 is a histone desuccinylase that functionally links to chromatin compaction and genome stability," *Nature Communications*, vol. 7, Article ID 12235, 2016.

[48] J. Yu, M. Haldar, S. Mallik, and D. K. Srivastava, "Role of the substrate specificity-defining residues of human SIRT5 in modulating the structural stability and inhibitory features of the enzyme," *PLoS One*, vol. 11, no. 3, Article ID e0152467, 2016.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 9251980, 11 pages
https://doi.org/10.1155/2020/9251980

Review Article

Epigenetic Modifications due to Environment, Ageing, Nutrition, and Endocrine Disrupting Chemicals and Their Effects on the Endocrine System

Elizabeth C. Plunk and Sean M. Richards

Department of Biological and Environmental Sciences, University of Tennessee, Chattanooga, TN 37403, USA

Correspondence should be addressed to Elizabeth C. Plunk; elizabeth.plunk27@gmail.com

Received 18 December 2019; Accepted 18 June 2020; Published 21 July 2020

Academic Editor: Kazuhiro Shiizaki

The epigenome of an individual can be altered by endogenous hormones, environment, age, diet, and exposure to endocrine disrupting chemicals (EDCs), and the effects of these modifications can be seen across generations. Epigenetic modifications to the genome can alter the phenotype of the individual without altering the DNA sequence itself. Epigenetic modifications include DNA methylation, histone modification, and aberrant microRNA (miRNA) expression; they begin during germ cell development and embryogenesis and continue until death. Hormone modulation occurs during the ageing process due to epigenetic modifications. Maternal overnutrition or undernutrition can affect the epigenome of the fetus, and the effects can be seen throughout life. Furthermore, maternal care during the childhood of the offspring can lead to different phenotypes seen in adulthood. Diseases controlled by the endocrine system, such as obesity and diabetes, as well as infertility in females can be associated with epigenetic changes. Not only can these phenotypes be seen in F1, but also some chemical effects can be passed through the germline and have effects transgenerationally, and the phenotypes are seen in F3. The following literature review expands upon these topics and discusses the state of the science related to epigenetic effects of age, diet, and EDCs on the endocrine system.

1. Introduction to Epigenetics

Epigenetic reprogramming occurs during germ cell development and embryogenesis, and epigenetic modifications influence the expression of genes, creating phenotypic expression, without changing the genetic sequence [1]. To date, there are three known epigenetic mechanisms of endocrine disruption: DNA methylation, histone modification, and aberrant miRNA expression. These mechanisms have myriad effects on human development, health, and reproduction.

Epigenetic modifications do not alter the gene sequence; however, they can alter gene expression [2]. Epigenetic changes are heritable [3] during cell division [4] and reversible based on environmental signals [5]. Endocrine Disrupting Compounds (EDCs) that methylate DNA can target the cytosine residues located in cytosine-phosphate-guanine (CpG) dinucleotides by adding a methyl group to the 5′ position of the cytosine pyrimidine ring [6]. The importance of methylation at CpG sites and gene expression varies. Gene expression is regulated by cytosine methylation as well as transcription factor binding [7]. It is generally accepted that DNA methylation does not *directly* affect the DNA molecule-with the exception of cytosine methylation at CpG sites. Cytosine methylation is required for embryonic development in mammals and thus is the only epigenetic modification known to directly affect the DNA molecule [5].

Histone modification often occurs concomitantly with DNA methylation and results in short- and long-term alterations in transcription programs [8]. Histones are proteins that pack DNA into nucleosomes which make up chromatin, and modifications in the histone can alter the accessibility of chromatin as well as altering the transcriptional activities in the cell [9]. Gene activation and silencing can be associated with histone modifications [10]. Histone

modifications can be responsible for the transduction of hormones such as insulin growth factor I (IGF1) [11].

The third type of epigenetic endocrine disruption is the aberrant expression of microRNAs (miRNAs). miRNAs are closely related to small interfering RNAs (siRNAs) which are involved in DNA methylation and histone modifications [9], and they are composed of 21–24 single stranded nucleotides [12]. miRNAs are noncoding RNA produced from introns/exons that bind to target mRNAs in order to suppress protein translation and posttranscriptional gene expression. Thus, if miRNA expression is amplified or diminished, production of protein or peptide hormones could be disrupted. miRNAs have broad specificity for mRNAs, and more than one miRNA can target mRNAs [13].

2. Hormonal Modulation and Endocrine System Plasticity through Epigenetic Mechanisms

The endocrine system is responsible for maintaining homeostasis in the body; therefore, it must be very responsive to environmental alterations [4]. The nutritional environment that the mother has during the last 3 months of pregnancy cues the infant's system as to what the environment will be once out of the womb [14]. The endocrine system will respond to this by altering the metabolic system based on the nutritional environment that the child is suspected to be exposed to [14]. However, this can lead to diseases in child and adulthood if incorrectly adjusted. The child can possibly experience health problems such as cardiovascular disease, diabetes, and obesity if this metabolic adjustment was erroneous [14]. Furthermore, maternal hormonal signals during pregnancy can modify the organizational pathways in the fetus' brain nuclei, which can affect physiological and behavioral responses in the adult offspring [15].

Hormones can affect the phenotype, typically of behavior [16], as well as regulating development, growth, reproduction, metabolism, and immunity [17]. The abundance of hormone receptors themselves can explain the differences between phenotypes among individuals when encountering specific stressors [18]. For example, corticosterone has two receptor types: one that is active when the hormone is present in low concentration (high affinity receptor) and the other that is active when the hormone is in high concentration (the low affinity receptor) [19]. The high affinity receptor is mediating the effects of corticosterone levels daily, and the low affinity receptors are engaged during responses to stress when cortisol is at greatest concentrations, for example [19].

Phenotypic plasticity occurs in response to internal and external environmental cues that lead to the cell changing its behavior [4]. Environmental stressors, both endogenous and exogenous, can partition the genome into active and inactive domains epigenetically, which can drive phenotype plasticity [3]. Critical time periods in epigenetic reprogramming are during gametogenesis and early preimplantation development, and genome-wide demethylation occurs upon fertilization [20]. Furthermore, germ cells and early embryonic cells have been the only cells affected by epigenetic programming on a genome-wide scale [20]. This phenomenon allows for epigenetic traits to be turned from "stable" to "flexible." Epigenetic programming is important for erasing genomic imprints and epimutations that could be inherited across generations. It also controls transposon silencing [20].

Ong et al. [21] studied the effects of exposing the central amygdala (CeA) to elevated corticosteroids (CORT) on anxiety like behaviors in mice. Researchers inserted pellets of CORT in the CeA in mice. They found that the elevated levels of CORT in the CeA decreased histone acetylation in histone 3 at lysine 9 (H3K9) as well as decreasing glucocorticoid receptors (GR) and increasing corticotropin-releasing factor (CRF) expression. The same researchers also implanted CORT into the dorsal margin of the CeA; GR expression was reduced in the CeA [21]. They also studied the role of histone acetylation in GR and CFR gene expression. In order to do this they treated animals with trichostatin A (TSA) after the CORT implantation. TSA reduces the effects of CORT-induced changes in gene expression. Ong et al. [21] also found that these animals showed greater GR expression in the CeA and showed a reduction in expression of CRF in the CeA. Increasing CORT in the CeA induced H3K9 deacetylation and inhibited histone deacetylases in the CeA and reduced anxiety-like behavior [21].

Ovarian functions such as folliculogenesis, oocyte maturation, ovulation, and luteal function are controlled by microRNA (miRNA) signalling [12]. Aberrant expression of miRNAs can alter endocrine functions. *Dicer* and *Drosha* are essential in the biogenesis of miRNAs [22, 23]. *DGCR8* is a cofactor of *DROSHA* [24]. At embryonic day (E) 6.5, the deletion of *DGCR8* led to the elimination of all miRNA in the embryo and resulted in embryo death [22]. After breeding heterozygously for *Drosha* +, embryos with *Drosha* -/- genotype were much smaller than heterozygous embryos at E6.5, and the embryos deteriorated between E7.5 and E8.5 [22]. A lack of *Drosha* does not cause infertility in aging female mice [25]. In contrast, female fertility in adulthood is not possible without oocyte *Dicer* expression [20]. *Ddx4-Dicer* conditional knockout (cKO) female mice were bred with fertile male mice, and no offspring were produced suggesting *Dicer* cKO leads to infertility [22]. At PND 120 ovaries were evaluated for status of follicles, and *Ddx4-Dicer* cKO ovaries had no developing follicles; interestingly, primordial, primary, secondary, and antral follicles were present at PND 30 and PND 40, suggesting a continuous loss of follicles with age [22].

Wang et al. [24] generated mouse *Dgcr8* knockout embryonic stem (ES) cells in order to assess the role of *Dgcr8* in miRNA processing as well as studying the role of miRNAs in early development. After creating *Dgcr8* knockout ES cells, no mature or intermediate pre-miRNAs were present [24]. Without *Dgcr8,* proper ES cell proliferation and cell-cycle progression cannot occur, and ES cell self-renewal cannot be silenced without miRNAs [24].

Epigenetic mechanisms affect fertility and hormonal responses within an organism and occur due to external and internal stimuli. These modifications and responses can

result in anxiety-like behaviors [21], and if the proteins within miRNAs are affected, fertility can be compromised [22].

3. Environmental Regulation of Endocrine Systems through Epigenetic Mechanisms

Three genomic targets have been identified as susceptible to environmental epigenetic changes: promoter regions of housekeeping genes, transposable elements that lie adjacent to genes with metastable epialleles, and regulatory elements of imprinted genes [2]. All three targets are rich in CpG dinucleotides, thus making them susceptible. These sequences can be unmethylated, methylated, or differentially methylated between organisms, and some have histone modifications in the same region which determines levels of gene expression [2]. Through environmental stimuli such as exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the methylation statuses of *H19* and *Igf2* are altered [26], and other environmental stimuli such as stress lead to the body up- or downregulating enzyme 11β-HSD2 which protects the fetus from maternal hormones [27]. The expression of these enzymes can also be altered due to exposure to metals or metalloids [28]. Likewise, maternal care can alter the response to stress later in life of mice by altering the DNA methylation status and chromatin structure [29].

Wu and colleagues [26] directly studied the effects that TCDD has on the methylation status of *H19* and *Igf2* which are growth-related imprinted genes. They exposed mice embryos preimplantation to TCDD and then implanted the embryo in unexposed female mice. With exposure to TCDD the expression level of *H19* decreased, and the expression of *Igf2* tended to decrease but not at a statistically significant value [26]. The 5-methylcytosine concentration in the targeted region of genomic DNA was higher in TCDD-exposed embryos compared to the control. The methylation level of *H19* and *Igf2* in the imprint control region was also higher in TCDD-exposed samples, and this was significantly correlated with lower fetal body weight [26].

Maternal behavior produces stable alterations in DNA methylation and chromatin structure which affects the gene expression in offspring [29]. In a mouse study, adult mice who had high pup licking and grooming (LG) and arched-back nursing (ABN) mothers as infants had a more moderate hypothalamic-pituitary-adrenal (HPA) function, controlled by the neuroendocrine system [30], respond to stress compared to the offspring of low LG-ABN mothers [29]. This outcome is due to the differences in hippocampal glucocorticoid receptor gene (GR) levels and GC negative feedback sensitivity [29]. Using sodium bisulfite mapping, the 5′ and 3′ CpG dinucleotides within the nerve growth factor-inducible protein-A (NGFI-A) binding site were heavily methylated in both the high and low LG-ABN offspring on postnatal day (PND) 1 due to the postnatal wave of new methylation [29]. In the high LG-ABN, the 5′ CpG dinucleotide of the NGFI-A binding site was demethylated by PND 6 [29]. In the offspring of low LG-ABN mothers, the 5′ CpG dinucleotide appears to be methylated always [29].

Even small differences in epigenetic patterns can have an impact on phenotype [31], and the environment can influence these. Monozygous twins begin with the same environment in the womb, and once they are born and throughout life their environment differs from one another. Differences in the genomic distribution of 5-methylcytosine DNA and histone acetylation play a role in gene expression in monozygous twins [32]. These changes can be attributed to both external and internal factors. Nearly one-third of monozygous twins have epigenetic differences in DNA methylation and histone modification. Fraga et al. [32] found that as the twins got older there were more differences in their epigenetic patterns.

Stress during childhood has been linked to reduced glucocorticoid receptors in adults. McGowan et al. [7] found that, in suicide completers who had a history of child abuse, the hippocampal expression of glucocorticoid receptor mRNA and glucocorticoid receptor 1_f splice variant was significantly decreased compared to suicide completers without a history of child abuse. Of note, the total glucocorticoid receptor expression is equal to its hippocampal expression. This difference was linked to the increase in methylation of the human glucocorticoid receptor gene (*NR3C1)* and reduced expression of the glucocorticoid receptor mRNA [7].

Glucocorticoids (GCs) are steroid hormones that regulate many functions such as blood pressure and metabolic processes in the body due to their ability to induce many genes' expressions throughout bodily systems [33]. The role of 1β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is to protect the fetus from the high levels of maternal GC hormones [28]. 11β-HSD2 activity and expression in the placental tissues can be altered by exposure to metals and metalloids [34]. The decrease of expression and activity of 11β-HSD2 will result in increased GC exposure and enhanced response to GC in the fetus which will cause GC-induced fetal growth restriction (FGR) [28]. Exposure to cadmium (Cd) through the environment or smoking can decrease 11β-HSD2 expression and activity in trophoblast cells in the placenta [28]. In contrast, Mikelson et al. [35] found that there was no correlation between 11β-HSD2 and placental concentrations of Cd. Co, Ni, Zn, Fe, and Cu were also evaluated, and there was a positive correlation between the concentration of these metals and the expression of 11β-HSD2 only in male placentae, whereas female placentae did not show any correlation between expression of 11β-HSD2 and concentration of these metals and metalloids [35].

11β-HSD2 activity and expression in the placental tissues can also be altered by maternal stress [27]. An upregulation of 11β-HSD2 in the placental tissues can be triggered by maternal acute exposure to stress on GD 20 [27]. In contrast, maternal chronic stress exposure from GD 14 to GD 19 did not alter 11β-HSD2 activity; however, it did reduce the ability to upregulate 11β-HSD2 activity during acute stress exposure [27].

Environmental factors that affect expression of various genes include exposure to TCDD, maternal behavior to the offspring, stress during childhood, and placental exposure to metals and metalloids. They, respectively, affect the

expression of growth imprinting genes *H19* and *Igf2,* GR, *NR3C1,* and 11β-HSD2.

4. Ageing-Induced Hormone Modulation and Epigenome Modifications

Hormone modulation occurs naturally as we age. GC expression levels increase as we age while the abundance of GC receptors decreases, which can in turn leads to more GC production [33]. In eukaryotic cells, modification of 5-methylcytosine is the most abundant DNA base modification that is responsible for gene repression [36]. During the process of aging, the level of 5-methylcytosine in DNA decreases leading to hypomethylation in most tissues while hypermethylation occurs in promoter regions [37]. As described above, histone modification and DNA methylation often occur concomitantly [8]. Histone modifications can alter the accessibility of chromatin, alter transcriptional activities in the cell [9], and cause transduction of some hormones [11].

The ubiquitin-proteasome system (UPS) deteriorates during the ageing process [38]. Histone deacetylase 1 (HDAC1), DNA cytosine-5-methyltransferase (DNMT1), and chromatin modifiers are regulated by the UPS. Moreover, while ageing, the number of histone chaperones, ASF1A/B, and chromatin assembly factor 1 (CAF1) decreases; therefore the assembly of histones around DNA and histone incorporation into chromatin are also decreased [39].

The role of corticosteroid binding globulins (CBG) is to transport GCs in the blood as well as regulating entry of GCs into the blood. There are no changes in CBG with age [33]. However, the activity of 11β-HSD1 enzymes, which convert inactive cortisone into active cortisol [40], increases with age. In contrast, 11β-HSD2 activity decreases with age which leads to the increased bioavailability of intracellular GCs. Unregulated GC bioavailability has been implicated in age-related disorders such as metabolic diseases, cognitive decline, and cardiovascular risk [41].

Global hypomethylation can be caused by a decrease in activity of DNMT1, which specifically adds methyl groups to cytosines [42]. Likewise, global hypomethylation can occur due to the inhibition of the activity of DNMT1 by cellular S-adenosylhomocysteine, which increases during aging [42]. Global DNA methylation can decrease due to the reduction of sex hormones during aging [42]. Histone acetylation is also altered during ageing. The irregular histone acetylation is due to the change in activity balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) that occurs with age. This change can increase the development of age-associated diseases such as insulin resistance [42].

Choi et al. [43] hypothesized that cumulative estrogen exposure across the lifetime may be associated with differential methylation of genes. The reproductive period in a female's life is measured from the age of menarche to the age of menopause, and during this time the woman is exposed to varying activities and types of estrogens [43]. Women with longer reproductive periods have more methylation across the oxidative phosphorylation (OXPHOS) apparatus, which are genes found mainly in autosomes. High levels of methylation in OXPHOS are associated with strokes [43]. Choi et al. [43] also tested their hypothesis by examining the association between reproductive period and DNA methylation in the 95 genes of the 5′ to 3′ UTR [43]. The transcription start site *NDUFS8* showed the most methylation in the OXPHOS apparatus [43]. In the second approach, they [43] used Wilcoxon rank-sum tests to analyze the Hallmark pathways from the Molecular Signatures Database [44] that were the most differentially methylated pathways between organisms in relation to the reproductive period [43]. Ten pathways were differentially methylated; the third most significantly methylated pathway was the Hallmark oxidative phosphorylation pathway [43]. In the Hallmark oxidative phosphorylation pathway, 95 OXPHOS genes overlapped from the first approach, and 68 genes were found to be common; however, 133 genes were unique to the Hallmark pathway [43].

During ageing histone H3.3 becomes more abundant. Histone H3.3 packages the neuronal genome looser than other histones [45]. The looser packaging allows for more histone exchange as well as increased access to transcription machinery [45] and, thus, more histone modification. In baboons, HIRA, a heterochromatin protein and H3.3 specific chaperone found also in humans, increases [46]. Likewise, in embryonic and postnatal development of chicken and mice, H3.3 levels increase in the brain, heart, kidney, liver, and spleen [47].

Epigenetic modifications such as hyper- and hypomethylation and histone modifications can be seen on certain genes through the ageing process.

5. Effects of Nutritional Exposure on Endocrine Systems through Epigenetic Route

Bioactive food components can inhibit enzymes that mediate DNA methylation and histone modifications [37]. Nutrients, such as folate, can act as methyl sources that contribute to the production of S-adenosylmethionine (SAM), a methyl donor for methylation reactions [37]. Once SAM donates a methyl group to a methylation reaction, it converts to S-adenosylhomocysteine (SAH), which is further converted to homocysteine [37]. A deficiency in nutrients that donate methyl groups can lead to the lowered availability of SAM and SAH, which decreases the frequency of DNA methylation, due to the decrease of expression of DNMT1 [37]. Epigenetic reductions of DNA methylation in the preovulatory oocyte can have long-term effects on the embryo [48]; furthermore, folic acid supplementation before conception has been linked to the imprinting status of *IGF2* [49].

Insulin-like growth factor 2 (I*GF2*) gene affects growth weight by encoding a fetal and placenta growth factor [50]. During the third trimester maternal concentrations of *IGF2* are inversely related to the mother's body weight and are positively correlated with the placental weight and newborn's height [50]. Lower *IGF2* concentrations during the

third trimester on the maternal placental side are correlated with higher *IFG2* differentially methylated region (DMR) 2 [50]. Increased methylation of the *IGF2* DMR is associated with periconceptional folic acid supplementation of the mother [49]. *IGF2* methylation and birth weight are inversely related [49]. 17 months after delivery the biomarkers, SAM, SAH, or SAM/SAH in the mother and child did not differ between periconceptional use of folic acid and no periconceptional use of folic acid [49].

In the weeks leading to conception, sheep were exposed to methyl deficient (MD) diet which included low levels of vitamin B_{12}, folate, and amino acid methionine [48]. The MD diet resulted in higher concentrations of homocysteine in ovarian follicular fluid, plasma, and granulosa cell lysates compared to the control diet [48]. The MD offspring showed a greater growth rate until weaning (3 months) compared to the control offspring, and this continued until 22 months of age resulting in heavier MD offspring, with the females being heavier than the males [48]. The body composition did not differ between the MD group and control group until 22 months when the MD males became fatter and had less muscle mass than the control males [48]. MD males were the only group to show insulin resistance, which was independent of adiposity [48]. The higher adiposity and insulin resistance in MD males can be explained by epigenetic modification [48]. Of 1,400 CpG sites analyzed, 57 loci were altered in two or more MD males in comparison to the controls, and 88% of the altered loci were unmethylated or hypomethylated in comparison to the controls [48]. Of the changed loci, 53% were specific to MD males, while only 12% were specific to MD females [48].

Godfrey et al. [51] assessed the methylation status of CpGs in the promoters of candidate genes in umbilical cord tissue collected at birth and the adiposity status of the children at 9 years of age. Of the 31 CpGs that showed hyper- and hypomethylation, 7 had significant associations with the child's adiposity and body fat distribution at 9 years of age [51]. Higher CpG methylation of *RXRA*, which is found among positive regulatory elements of transcription, in the umbilical cord was associated with lower maternal carbohydrate intake, and higher adiposity at 9 years of age [51].

Maternal under- and overnutrition during pregnancy and breast-feeding may affect infant genes that control lipid and carbohydrate metabolism, therefore, inducing alterations in epigenetic routes [52]. Heijmans et al. [53] studied whether periconceptional exposure to famine during the early stages of development is associated with differences in *IGF2* differentially methylated regions (DMR) in adults. Famine exposed individuals were compared to the same-sex siblings [53]. Four of the five CpG sites measured in the *IGF2* DMR showed to be significantly less methylated in individuals exposed to famine periconceptionally compared to their same-sex siblings [53]. Periconceptional exposure to famine was associated with 5.2% lower methylation with no sex-dependent difference [53].

Bogdarina et al. [54] provided pregnant rats with either 20% protein rat chow, which accounts for normal protein consumption (control), or 8% protein rat chow which accounts for maternal low protein (MLP) [54]. This diet was given from pregnancy to weaning which occurred at 3 weeks of age of offspring. Liver, lung, kidney, brain, heart, and adrenal gland tissues were harvested and analyzed at week 1 and week 12. In MLP female offspring at 1 and 12 weeks there was an increase in expression of AT_{1a} angiotensin receptor in the kidney. In male and female MLP offspring, there was an increase in AT_{1b} angiotensin receptor in the adrenal gland, which is associated with the development of hypertension [54]. However, a decrease in expression was found in the AT_2 receptor in MLP offspring at 1 and 12 weeks. In the liver, angiotensinogen and AT_{1a} receptors showed increased expression at week 1, but the expression normalized by week 12. The methylation status of the 17 CpG sites in the AT_{1a} promoter was evaluated, and no difference was found between the methylation frequency of this region of control offspring and the MLP offspring [54].

Nutritional intake by the mother can lead to hyper- or hypomethylation of genes important to fetal development, and the effects of these epigenetic modifications can be seen after infancy.

6. Endocrine Disruptors and Endocrine Responses through Epigenetic Routes

Endocrine disrupting chemicals (EDCs) have a variety of mechanisms. They are structural similarity to hormones, and their mechanisms include altering normal hormone concentrations, inhibiting or stimulating the production and metabolism of hormones, or changing hormones' movement through the body [55]. These actions can result in the production of adverse developmental, reproductive, neurological, and immune effects in humans [17]. EDCs can cause effects at low doses in a tissue-specific manner, and the age at which a person is exposed to EDCs can determine their effects [55]. Prenatal exposure can lead to reproductive pathologies [55], neurodevelopmental delays in children [17], and metabolic and hormonal disorders later in life by altering normal cellular and tissue development and function during developmental programming [55].

Androgens and estrogens, steroid hormones, are involved in normal growth and development of human secondary sex organs [56]. Three types of estrogen receptors that EDCs can interact with are nuclear estrogen receptors (ERα and ERβ) which are essential in transcription regulation [57], membrane bound estrogen receptors, and estrogen G protein-coupled receptor (GPR30) [55]. Xenoestrogens can function by utilizing membrane bound receptors and second messenger pathways, an indirect pathway [58], as well as disrupting normal signalling pathways [59]. Indirect pathways can be activated at low xenoestrogen concentrations and can lead to nongenomic effects on gene expression [58]. The nongenomic effects can be perpetuated by continuous ligand stimulation and sending signals downstream, which can cause genomic effects once the signals are in a position to control the activation state of transcription factors [59].

Anderson et al. [57] analyzed the relationship between nuclear receptors and histone methylation modifiers in embryonic testis tissue in mice. Nuclear receptor *Rarb* and

methyltransferase *Suv39h1* are both present in similar expression concentration in efferent ducts, epididymis, and vas deferens in embryonic tissue; likewise, nuclear receptor *Nr1h2* and methyltransferase *Suv420h2* show a similar relationship [57]. A strong correlated expression is present in the embryonic tissue from efferent ducts, epididymis, and vas deferens between the nuclear receptor *Rarb* and methyltransferase *Suv39h1* as well as between the nuclear receptor *Nr1h2* and the methyltransferase *Suv420h2* [57].

Histone modification can act as a gatekeeper mechanism by promoting or preventing the promoter access to liganded-nuclear receptors [60]. The gatekeeper theory may explain the differing phenotypes displayed when the same genes with diverse functional roles are exposed to EDCs [57]. The gatekeeper concept was studied in genes in testis by exposing rats from gestational day (GD) 6 to PND 92 to low and high doses of myclobutanil, propiconazole, and triadimefon. All are endocrine disrupting fungicides. Anderson et al. [57] determined the gatekeeper set to be the methyltransferases: *Ehmt1, Ehmt2, Prdm2,* and *Setdb1*. The expression of the receptor genes *Ar* (androgen receptor gene) and *Esr2* (estrogen receptor gene) was studied in relation to the expression of the identified gatekeeper methyltransferases. In the samples exposed to high doses of triadimefon, the gatekeeper set had highly correlated expression with the receptors. In the 22 phenotypic genes studies, nine did not show coexpression with either *Ar* or *Esr2*. Three had coexpression approaching but not reaching significance with *Esr2*. Ten showed statistically significant coexpression with *Esr2, Ar,* or both. In contrast, only two samples exposed to low dose of propiconazole had two gatekeeper genes showing high coexpression with each other, and none showed coexpression with a nuclear receptor, which is important in transcription regulation. These results support the concept of the gatekeeper mechanism where histone methylation modifiers work in unison with nuclear receptors to mediate transcriptional change in target genes [57].

Brominated flame retardants (BFRs) are *in vitro* and *in vivo* endocrine disruptors [61] that structurally resemble polychlorinated bisphenyls (PCB) [62]. There are many BFR congeners that affect thyroid hormones, and polybrominated diphenyl ether (PBDE) congeners affect spermatogenesis at doses as low as 60 ug/kg/bw [61]. BFRs can also bind to estrogen receptors [62]. Kamstra et al. [63] studied the effects of the BFR 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47) on 3T3-L1 adipocyte differentiation on *in vitro* adipocytes. After 8 days of exposure to BDE-47, they [63] found demethylation of CpG regions and increased gene expression of *Ppary2,* peroxisome proliferator activated receptor, and gamma 2. Kamstra and colleagues found significant demethylation of the 3 CpG regions between base pairs −337 and −192. The reason for increased gene expression of *Ppary2* is due to decreased methylation in the *Ppary2* promoter [63].

Exposure to endocrine disruptors such as fungicides and BFRs can affect the expression of various receptors due to differential methylation and histone modifications.

7. Epigenetic Transgenerational Inheritance of Endocrine Diseases Promoted by Ageing, Diet, and Environmental Endocrine Disruptors

Sex-steroid hormones establish methylation status during critical developmental periods [64], and epigenetic modifications begin as early as germ cell development and embryogenesis [1]. EDCs can act on sex-steroid hormone receptors, so during critical developmental periods if the fetus is exposed to EDCs, remethylation could occur within the germ cells, and these effects can be observed in subsequent generations [64]. Transgenerational effects, involving the transmission of epigenetic changes in the germline, occur when effects from the endocrine disruptor are observed without direct exposure, or in F3 [65, 66]. In contrast, if passed down by the paternal lineage, the epigenetic phenotype becomes transgenerational once expressed in the F2 generation [66]. During adult life, if the F0 generation is exposed to endocrine disruptors, preconceptionally the F1 generation is being directly exposed [65]. Multigenerational effects are when any effects of the endocrine disruptor are observed in the F1 and F2 generations [65]. Some examples of environmental EDCs that can cause transgenerational effects are bisphenol-A (BPA), Di(2-ethylhexyl) phthalate (DEHP), and vinclozolin [65] (Table 1).

Vinclozolin is a fungicide known for its antiandrogenic endocrine disruption action [75]. Vinclozolin was the first EDC shown to display transgenerational inheritance [76]. Nilsson et al. [77] transiently exposed pregnant rats to vinclozolin, DDT, or control (DMSO) during GD 8–14. There was no increase of the ovarian diseases polycystic ovarian syndrome and primary ovarian insufficiency, in F1 and F2 generations from exposure to DDT and vinclozolin in mice, but there was an increase in the two ovarian diseases in F3 generation [77]. After ancestral exposure to DDT and vinclozolin, changes in DNA methylation in the F3 generation were present in the areas of the genome with relatively low CpG density [77]. Similarly, maternal exposure to diethylstilbestrol (DES) at a dose of 10 μg/kg/maternal body weight results in increased proliferative lesions (PPL) in the oviduct in F2 mice [67].

Inawaka et al. [68] examined whether the antiandrogens vinclozolin, procymidone, or flutamide caused transgenerational effects of DNA methylation in male rats. These researchers exposed maternal mice from GD 8 to GD 15 to 100 mg/kg/day vinclozolin, 100 mg/kg/day procymidone, or 10 mg/kg/day flutamide. DNA methylation analysis on 210 base pairs including 7 CpG sites in the *lysophospholipase* gene on F1 male pups occurred on PND 6. Exposed F1 males not sacrificed on PND 6 were bred with untreated-females, and subsequent DNA methylation analysis occurred on F2 generation on PND 6. DNA methylation status was comparable to the control, and no transgenerational effects were observed due to the DNA methylation caused by antiandrogens exposure to the F1 males [68].

Bisphenol-A (BPA) is a synthetic compound used in plastics and resins. Exposure to 5 mg/kg BPA prenatally

Table 1: EDCs and their effects as discussed in Section 7.

EDC	Animal model	Sex	Dose	Effect	Generation	Reference
DES	Mouse	Female	10 μg/kg/maternal bw	Increased PPL of oviduct	F2	[67]
Vinclozolin	Rat	Male	100 mg/kg/day	No effect on methylation	N/A	
Procymidone	Rat	Male	100 mg/kg/day	No effect on methylation	N/A	[68]
Flutamide	Rat	Male	10 mg/kg/day	No effect on methylation	N/A	
	Mice	Female	5mg/kg	Increased Meg3	F3	[65]
	Mice	Female	0.5, 20, 50 μg/kg/day	Dysregulated gene expression of ovarian apoptotic factors, oxidative stress factors, autophagy factors	N/A	[69]
BPA	Zebrafish	Female	20 μg/L	Variations in transcript abundance of genes *Iesr, star, lhcgr, fshr, amh*	Up to F3	[70]
	Rats	Male	1.2 μg and 2.4 μg	Reduced sperm count and motility, decreased ERβ expression	Up to F3	[71]
				Increased ERα expression	F1	
			1.2 μg	Decreased *Ar* expression	Up to F3	
			2.4 μg	Decreased *Ar* expression	Only F3	
			20 μg/kg/d	Ovarian weight decrease	F1	
			500 and 750 mg/kg/d	Uterine weight increase	F1	
			200 μg/kg/d	Decreased body weight	F3	
			20 and 200 μg/kg/d, 500 mg/kg/d	Decreased ovarian weight	F3	
			20 and 200 μg/kg/d and 500 and 750 mg/kg/d	Decreased liver weight	F3	
			750 mg/kg/d	Increased time in proestrus and metestrus/diestrus	F1	
			20 and 200 μg/kg/d	Decreased time in proestrus	F3	
			200 μg/kg/d	Increased time in estrus and metestrus/diestrus	F3	
			500 mg/kg/d and 750 mg/kg/d	Increased time in metestrus/diestrus	F3	
DEHP	Mice	Female	500 mg/kg/d	Decreased time in proestrus and estrus	F3	[72]
			750 mg/kg/d	Decreased number of primordial follicles	F1	
			20 μg/kg/d	Decreased number of preantral follicles	F1	
			200 μg/kg/d 500 mg/kg/d	Increased number of primordial follicles	F2	
			200 μg/kg/d	Increased number of primary follicles	F2	
			500 mg/kg/d	Increased levels of estradiol and decreased levels of testosterone	F1	
			750 mg/kg/d	Increased levels of estradiol	F1	
			20 μg/kg/d	Decreased testosterone and increased levels of estradiol	F2	
			200 μg/kg/d	Decreased progesterone	F2	
			500 mg/kg/d	Decreased testosterone	F2	
		Male	150 mg/kg	Lighter seminal vesicles	N/A	[73]
			200 mg/kg	Behavioral differences	N/A	
				Reduced kidney weight	F3	
TCDD	Rats	Female	N/A	Increased primordial follicle loss and polycystic ovarian disease	F1 & F3	[74]
		Male	N/A	Reduced kidney weight and increase in serum testosterone concentrations	F3	

disrupts the number of ER α-cells in brain regions that play a role in reproductive function in female mice [65]. Likewise, exposure to the same dose perinatally increases Meg3, an epigenetic modifier, expression in the female hypothalamus in F3 generation females. Increased concentrations of BPA and subsequent elevated expression of Meg3 are associated with precocious puberty in women and laboratory mice [65]. For example, in mice, ancestral exposure to BPA at 0.5, 20, and 50 μg/kg/day proved to cause dysregulated gene expression of ovarian apoptotic factors, oxidative stress factors, and autophagy factors [69]. The exposure dose of 5 mg/kg in mice is estimated to be what is present in human maternal blood, 0.3–18.9 ng/mL, making this dose environmentally relevant [78–80].

Furthermore, in a study examining the transgenerational effects of an environmental dose of 20 μg/L of BPA in female zebrafish, alterations in genes involved in female reproduction at a transcriptional level were found [70].

Variation in transcript abundance for the genes *esr, star, lhcgr,* and *fshr* was observed through F3 [70]. The transcript abundance of *amh,* a gene involved in gonadal differentiation, was reduced up to F3 due to hypermethylation of its promoter regions as well as alterations in H3K4me3/H3K27me3 [70].

In male rats, the lowest effective doses of BPA to reduce male fertility are 1.2 and 2.4 μg/kg bw [71]. Exposure to 1.2 μg and 2.4 μg BPA perinatally led to significantly reduced sperm count and sperm motility in F1, F2, and F3 males [71]. A decrease in ERβ was observed in all generations of both exposure groups, while in both F1 generations ERα expression was increased [71]. *Ar* expression was decreased in F1, F2, and F3 males in the 1.2 μg group, while in the 2.4 μg group a decrease was only seen in F3 [71]. The altered phenotype caused by perinatal exposure to BPA is seen transgenerationally; therefore, BPA exposure perinatally possibly caused reprogramming in the epigenome of the germ cells [81].

DEHP is a plasticizer found in numerous consumer products and is associated with transgenerational epigenetic effects in the ovaries of mice. Brehm et al. [72] studied the transgenerational effects of DEHP exposure at doses 20 μg/kg/d, 200 μg/kg/d, 500 mg/kg/d, and 750 mg/kg/d. Beginning on GD 11, the pregnant females were dosed with a solution of DEHP orally. Prenatal exposure to DEHP did not affect body weight or liver weight in the F1 and F2 generations [72]. In the F2 and F3 generations uterine weight was not affected by DEHP exposure. In the F1 generation ovarian weight decreased at a dose of 20 μg/kg/d, and uterine weight increased at 500 and 750 mg/kg/d doses. In the F3 generation, transgenerational effects observed were decreased body weight (200 μg/kg/d), decreased ovarian weight (20 and 200 μg/kg/d and 500 mg/kg/d), and decreased liver weight (20 and 200 μg/kg/d and 500 and 750 mg/kg/d) [72]. In the F1 generation, Brehm et al. [72] found that 750 mg/kg/d DEHP increased the time spent in proestrus and metestrus/diestrus while decreasing the time spent in estrus. There were no observable effects in the F2 generation. In F3 a dose of 20 μg/kg/d decreased the time spent in proestrus; likewise, exposure to 200 μg/kg/d of DEHP decreased the time spent in proestrus and increased the time spent in estrus and metestrus/diestrus [72]. Exposure to 500 mg/kg/d decreased the time spent in proestrus and estrus while increasing the time spent in metestrus/diestrus. Finally, 750 mg/kg/d increased the time spent in metestrus/diestrus [72]. Up to F3 reduced ovarian follicular reserve and oocyte and blastocyst developmental competence can be seen [82]. DEHP also affected the expression of genes responsible for trophoblast differentiation and implantation until F4 in mice [82]. The expression of *Lif-R* was upregulated in F2 and F3 generations with a dose of 0.05 and 5 mg DEHP/kg/day, and, in F4 *Lif-R*, it was upregulated due to dose of 0.5 mg/kg/day [82].

Male mice exposed to 150 mg/kg DEHP perinatally led to lighter seminal vesicles transgenerationally which suggests decreased testosterone levels [83]. In exposures of 200 mg/kg, the male mice showed behavior differences in comparison to the controls, which can be due to differing corticosterone levels [83].

Brehm et al. [72] also studied the effects of DEHP exposure on folliculogenesis which is possibly due to DNA methylation. In the F1 generation, they found that a dose of 750 mg/kg/d decreased the number of primordial follicles, and, at a dose of 20 μg/kg/d, the number of preantral follicles was decreased. In the F2 generation, the number of primordial follicles was increased at a dose of 500 mg/kg/day and the number of primary follicles increased at 200 μg/kg/d [72]. One transgenerational effect was observed at 200 μg/kg/d and the effect was the increase of the number of primordial follicles [72]. In the F1 generations, 500 mg/kg/d of DEPH increased the levels of estradiol and decreased the levels of testosterone, and 750 mg/kg/d increased the levels of estradiol in mice [72]. In the F2 generation, 20 μg/kg/d decreased testosterone and 200 ug/kg/d decreased progesterone [72]. The transgenerational effects were seen in 20 μg/kg/d when levels of estradiol increased and levels of testosterone decreased [72]. Testosterone decrease was also seen at a dose of 500 mg/kg/d [72].

Exposure to phthalate diethylhexyl phthalate in midgestation causes puberty delay in F1 and F3 generation males [73]. Other phenotypes in the F3 generation include lower sperm counts, testicular germ cell function, and increased incidence of abnormal seminiferous tubules [73].

Manikkam et al. [74] studied the transgenerational effects of TCDD. F0 generation female rats were exposed to TCDD from fetal days 8 to 14; then they were bred to produce F1 [74]. Only F1 and F3 generation adult rats were evaluated [74]. In F3 females, the body, ovarian, and uterine weights showed no change, while the kidney weight was reduced [74]. In F3 males, testis, epididymis, and prostate weights did not change, while kidney weight was reduced [74]. Serum testosterone concentrations were increased in F3 males, and serum estradiol concentration in F3 females during proestrus-estrus phase or diestrus phase showed no change [74]. These results led to the conclusion that F3 males experienced endocrine alterations while F3 females did not [74]. F1 and F3 females show an increase in primordial follicle loss and polycystic ovarian disease, and in F3 male sperm 50 differentially DNA methylated regions in promoters were found [74]. Overall, 50 statistically significant differentially DNA methylated regions in promoters between F3 males sperm epigenome were found [74].

Fungicides, BPA, DEHP, and TCDD affect reproduction transgenerationally in both males and females due to epigenetic modifications.

8. Conclusion

Epigenetic modifications due to exposure to different nutrients pre- and postnatally, EDCs, maternal behavior, and ageing can lead to various endocrine phenotypes. The endocrine system is susceptible to changes in the environment due to its role in maintaining homeostasis. *In utero*, the endocrine system predicts the environment the fetus will be living in, and the epigenetic reprogramming, if wrong, can lead to diseases such as cardiovascular disease, obesity, and diabetes. Hyper- and hypomethylation while ageing is responsible for the alterations in concentrations of hormones,

hormone receptors, and DNMTs. Maternal exposure to stress, metals, or metalloids can alter the expression of 11β-HSD2 in the placenta leaving the fetus unprotected from maternal GCs. EDCs such as fungicides, BPA, DEHP, and TCDD have transgenerational effects, seen in F3 (Table 1). In contrast, DES and antiandrogens only have multigenerational effects, seen in F2. Further research is needed to explore whether the concentrations in which humans are exposed to various EDCs cause epigenetic effects. The field of epigenetics and the effects on the endocrine system is growing, and more research is needed to see if the alterations in gene expression is solely due to epigenetic modifications or if other mechanisms are at play.

Abbreviations

ABN: Arched-back nursing
BFRs: Brominated flame retardants
BPA: Bisphenol-A
CAF1: Chromatin assembly factor 1
CBG: Corticosteroid binding globulins
CpG: Cytosine-phosphate-guanine
cKO: Conditional knockout
DEHP: Di(2-ethylhexyl) phthalate
DNMT1: DNA cytosine-5-methyltransferase
DES: Diethylstilbestrol
EDC: Endocrine disrupting chemical
ES: Embryonic stem
GCs: Glucocorticoids
GR: Glucocorticoid receptor gene
HDAC1: Histone deacetylase 1
HPA: hypothalamic-pituitary-adrenal
IGF2: Insulin-like growth factor 2
LG: Licking and grooming
MD: Methyl deficient
MLP: Maternal low protein
miRNA: MicroRNA
PBDE: Polybrominated diphenyl ether
PCB: Polychlorinated bisphenyls
PND: Postnatal day
SAH: S-Adenosylhomocysteine
Sam: S-Adenosylmethionine
TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin
UPS: Ubiquitin-proteasome system.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] C. Dupont, D. Armant, and C. Brenner, "Epigenetics: definition, mechanisms and clinical perspective," *Seminars in Reproductive Medicine*, vol. 27, no. 5, pp. 351–357, 2009.

[2] R. L. Jirtle and M. K. Skinner, "Environmental epigenomics and disease susceptibility," *Nature Reviews Genetics*, vol. 8, no. 4, pp. 253–262, 2007.

[3] X. Zhang and S.-M. Ho, "Epigenetics meets endocrinology," *Journal of Molecular Endocrinology*, vol. 46, no. 1, pp. R11–R32, 2011.

[4] A. P. Feinberg, "Phenotypic plasticity and the epigenetics of human disease," *Nature*, vol. 447, no. 7143, pp. 433–440, 2007.

[5] A. Gabory, L. Attig, and C. Junien, "Epigenetic mechanisms involved in developmental nutritional programming," *World Journal of Diabetes*, vol. 2, no. 10, pp. 164–175, 2011.

[6] S. K. T. Ooi, A. H. O'Donnell, and T. H. Bestor, "Mammalian cytosine methylation at a glance," *Journal of Cell Science*, vol. 122, no. 16, pp. 2787–2791, 2009.

[7] P. O. McGowan, A. Sasaki, A. C. D'Alessio et al., "Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse," *Nature Neuroscience*, vol. 12, no. 3, pp. 342–348, 2009.

[8] Y. Kondo, "Epigenetic cross-talk between DNA methylation and histone modifications in human cancers," *Yonsei Medical Journal*, vol. 50, no. 4, pp. 455–463, 2009.

[9] J. C. Chuang and P. A. Jones, "Epigenetics and MicroRNAs," *Pediatric Research*, vol. 61, no. 5 Part 2, pp. 24R–29R, 2007.

[10] C. R. Clapier and B. R. Cairns, "The biology of chromatin remodeling complexes," *Annual Review of Biochemistry*, vol. 78, no. 1, pp. 273–304, 2009.

[11] L. Y. Sun and A. J. D'Ercole, "Insulin-like growth factor-I stimulates histone H3 and H4 acetylation in the brainin vivo," *Endocrinology*, vol. 147, no. 11, pp. 5480–5490, 2006.

[12] P. Brachova, W. T. Hung, L. K. McGinnis, and L. K. Christenson, "MicroRNA regulation of endocrine functions in the ovary," in *Post-transcriptional Mechanisms in Endocrine Regulation*, PhD. K. Menon and PhD. A. Goldstrohm, Eds., Springer, Berlin, Germany, 2016.

[13] I. G. Cannell, Y. W. Kong, and M. Bushell, "How do microRNAs regulate gene expression?" *Biochemical Society Transactions*, vol. 36, no. 6, pp. 1224–1231, 2008.

[14] P. Bateson, "Fetal experience and good adult designa," *International Journal of Epidemiology*, vol. 30, no. 5, pp. 928–934, 2001.

[15] J. Lindström, "Early Development and fitness in birds and mammals," *Trends in Ecology & Evolution*, vol. 14, no. 9, pp. 343–348, 1999.

[16] A. P. Arnold, "The effects of castration and androgen replacement on song, courtship, and aggression in zebra finches (Poephila guttata)," *Journal of Experimental Zoology*, vol. 191, no. 3, pp. 309–325, 1975.

[17] C. Monneret, "What is an endocrine disruptor?" *Comptes Rendus Biologies*, vol. 340, no. 9-10, pp. 403–405, 2017.

[18] R. A. Waterland and K. B. Michels, "Epigenetic epidemiology of the developmental origins hypothesis," *Annual Review of Nutrition*, vol. 27, no. 1, pp. 363–388, 2007.

[19] E. R. De Kloet, E. Vreugdenhil, M. S. Oitzl, and M. Joels, "Brain corticosteroid receptor balance in health and disease," *Endocrine Reviews*, vol. 19, no. 3, pp. 269–301, 1998.

[20] Z. Hochberg, R. Feil, M. Constancia et al., "Child health, developmental plasticity, and epigenetic programming," *Endocrine Reviews*, vol. 32, no. 2, pp. 159–224, 2011.

[21] L. Ong, J. Schulkin, C. O. Ligon, and B. Greenwood-van Meerveld, "Epigenetic modulation of chronic anxiety and pain by histone deacetylation," *Molecular Psychiatry*, vol. 20, no. 10, pp. 1219–1231, 2015.

[22] S. Yuan, N. Ortogero, Q. Wu, H. Zheng, and W. Yan, "Murine follicular development requires oocyte DICER, but not DROSHA," *Biology of Reproduction*, vol. 91, pp. 1–8, 2014.

[23] E. Bernstein, S. Y. Kim, M. A. Carmell et al., "Dicer is essential for mouse development," *Nature Genetics*, vol. 35, no. 3, pp. 215–217, 2003.

[24] Y. Wang, R. Medvid, C. Melton, R. Jaenisch, and R. Blelloch, "DGCR8 is essential for microRNA biogenesis and silencing

of embryonic stem cell self-renewal," *Nature Genetics*, vol. 39, no. 3, pp. 380–385, 2007.

[25] S. Yuan, N. Ortogero, Q. Wu, H. Zheng, and W. Yan, "Murine follicular development requires oocyte DICER, but not DROSHA," *Biology of Reproduction*, vol. 91, no. 2, pp. 1–8, 2014.

[26] Q. Wu, S. Ohsako, R. Ishimura, J. S. Suzuki, and C. Tohyama, "Exposure of mouse preimplantation embryos to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the methylation status of imprinted genes H19 and Igf21," *Biology of Reproduction*, vol. 70, no. 6, pp. 1790–1797, 2004.

[27] L. A. M. Welberg, K. V. Thrivikraman, and P. M. Plotsky, "Chronic maternal stress inhibits the capacity to up-regulate placental 11-hydroxysteroid dehydrogenase type 2 activity," *Journal of Endocrinology*, vol. 186, no. 3, pp. R7–R12, 2005.

[28] K. Yang, L. Julan, F. Rubio, A. Sharma, and H. Guan, "Cadmium reduces. 11β-hydroxysteroid dehydrogenase type 2 activity and expression in human placental trophoblast cells," *American Journal of Physiology*, vol. 290, 2006.

[29] I. C. G. Weaver, N. Cervoni, F. A. Champagne et al., "Epigenetic programming by maternal behavior," *Nature Neuroscience*, vol. 7, no. 8, pp. 847–854, 2004.

[30] I. C. G. Meaney, "Epigenetic effects of glucocorticoids," *Seminars in Fetal and Neonatal Medicine*, vol. 14, no. 3, pp. 143–150, 2009.

[31] D. Humphreys, K. Eggan, H. Akutsu et al., "Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei," *Proceedings of the National Academy of Sciences*, vol. 99, pp. 12889–12894, 2002.

[32] M. F. Fraga, E. Ballestar, M. F. Paz et al., "From the Cover: epigenetic differences arise during the lifetime of monozygotic twins," *Proceedings of the National Academy of Sciences*, vol. 102, no. 30, pp. 10604–10609, 2005.

[33] B. Wu and R. Sharma, "Glucocorticoid hormones in aging," in *Hormones in Ageing and Longevity*, S. Rattan and R. Sharma, Eds., vol. 6, Berlin, Germany, Springer, 2017.

[34] A. M. Ronco, E. Llaguno, M. J. Epuñan, and M. N. Llanos, "Effect of cadmium on cortisol production and 11β-hydroxysteroid dehydrogenase 2 expression by cultured human choriocarcinoma cells (JEG-3)," *Toxicology in Vitro*, vol. 24, no. 6, pp. 1532–1537, 2010.

[35] C. Mikelson, M. J. Kovach, J. Troisi et al., "Placental 11β-Hydroxysteroid dehydrogenase type 2 expression: correlations with birth weight and placental metal concentrations," *Placenta*, vol. 36, no. 11, pp. 1212–1217, 2015.

[36] M. Tang and K. Rai, "Computational analysis of epigenetic modifications in melanoma," *Computational Epigenetics and Diseases*, vol. 9, pp. 327–342, 2019.

[37] K. C. Rai and S. W. Choi, "Nutritional epigenetics and aging," in *Nutrition, Exercise and Epigenetics: Ageing Interventions*, B. Yu, Ed., Vol. vol 2, Springer, Berlin, Germany, 2015.

[38] E. N. Tsakiri and I. P. Trougakos, "Chapter five- the amazing ubiquitin-proteasome system: structural components and implication in aging," in *International Review of Cell and Molecular Biology*, pp. 171–237, Elsevier, Amsterdam, Netherlands, 2015.

[39] C. M. Hammond, C. B. Strømme, H. Huang, D. J. Patel, and A. Groth, "Histone chaperone networks shaping chromatin function," *Nature Reviews Molecular Cell Biology*, vol. 18, no. 3, pp. 141–158, 2017.

[40] C. L. McTernan, N. Draper, H. Nicholson et al., "Reduced placental 11-hydroxysteroid dehydrogenase type 2 mRNA levels in human pregnancies complicated by intrauterine growth restriction: an analysis of possible mechanisms," *Journal of Clinical Endocrinology & Metabolism*, vol. 86, no. 10, pp. 4979–4983, 2001.

[41] K. Chapman, M. Holmes, and J. Seckl, "11β-Hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action," *Physiological Reviews*, vol. 93, no. 3, pp. 1139–1206, 2013.

[42] K. C. Kim and S.-W. Choi, "Nutritional epigenetics and aging," *Healthy Ageing and Longevity*, vol. 2, pp. 1–28, 2015.

[43] R. Choi, E. Patrick, C. M. Aubin et al., "Reproductive period and epigenetic modifications of the oxidative phosphorylation pathway in the human prefrontal cortex," *PLoS One*, vol. 13, no. 7, 2018.

[44] A. Subramanian, P. Tamayo, V. K. Mootha et al., "Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles," *Proceedings of the National Academy of Sciences*, vol. 102, no. 43, pp. 15545–15550, 2005.

[45] C. Das and J. K. Tyler, "Histone exchange and histone modifications during transcription and aging," *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, vol. 1819, pp. 332–342, 2012.

[46] J. C. Jeyapalan, M. Ferreira, J. M. Sedivy, and U. Herbig, "Accumulation of senescent cells in mitotic tissue of aging primates," *Mechanisms of Ageing and Development*, vol. 128, no. 1, pp. 36–44, 2007.

[47] M. K. Urban and A. Zweidler, "Changes in nucleosomal core histone variants during chicken development and maturation," *Developmental Biology*, vol. 95, no. 2, pp. 421–428, 1983.

[48] K. D. Sinclair, C. Allegrucci, R. Singh et al., "DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status," *Proceedings of the National Academy of Sciences*, vol. 104, no. 49, pp. 19351–19356, 2007.

[49] R. P. Steegers-Theunissen, S. A. Obermann-Borst, K. Dennis, L. Jan, and S. Cissy, "Periconceptional maternal folic acid use of 400 ug per day is related to increased methylation of the IGF2 gene in the very young child," *PLoS One*, vol. 4, 2009.

[50] J. St-Pierre, M.-F. Hivert, P. Perron et al., "IGF2 DNA methylation is a modulator of newborn's fetal growth and development," *Epigenetics*, vol. 7, no. 10, pp. 1125–1132, 2012.

[51] K. M. Godfrey, A. Sheppard, P. D. Gluckman et al., "Epigenetic gene promoter methylation at birth is associated with child's later adiposity," *Diabetes*, vol. 60, no. 5, pp. 1528–1534, 2011.

[52] R. B. Canani, M. Di Costanzo, L. Leone et al., "Epigenetic mechanisms elicited by nutrition in early life," *Nutrition Research Reviews*, vol. 24, no. 2, pp. 198–205, 2011.

[53] B. T. Heijmans, E. W. Tobi, A. D. Stein et al., "Persistent epigenetic differences associated with prenatal exposure to famine in humans," *Proceedings of the National Academy of Sciences*, vol. 105, no. 44, pp. 17046–17049, 2008.

[54] I. Bogdarina, S. Welham, P. J. King, S. P. Burns, and A. J. L. Clark, "Epigenetic modification of the renin-angiotensin system in the fetal programming of hypertension," *Circulation Research*, vol. 100, no. 4, pp. 520–526, 2007.

[55] T. T. Schug, A. Janesick, B. Blumberg, and J. J. Heindel, "Endocrine disrupting chemicals and disease susceptibility," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 127, no. 3–5, pp. 204–215, 2011.

[56] G. P. Veer Reddy, E. Cifuentes, M. Menon, and E. R. Barrack, "Steroid hormone and growth factor interaction in the regulation of cell cycle progression," *Steroid Hormones and Cell Cycle Regulation*, Springer, Berlin, Germany, pp. 111–135, 2002.

[57] A. M. Anderson, K. W. Certer, D. Anderson, and M. J. Wise, "Coexpression of nuclear receptors and histone methylation modifying genes in the testis: implications for endocrine disruptor modes of action," *PLoS One*, vol. 7, no. 4, 2012.

[58] B. Blumberg, T. Iguchi, and A. Odermatt, "Endocrine disrupting chemicals," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 127, no. 1-2, pp. 1–3, 2011.

[59] C. S. Watson, Y.-J. Jeng, and J. Guptarak, "Endocrine disruption via estrogen receptors that participate in nongenomic signaling pathways," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 127, no. 1-2, pp. 44–50, 2011.

[60] I. Garcia-Bassets, Y. S. Kwon, F. Telese et al., "Histone methylation-dependent mechanisms impose ligang dependency for gene activation by nuclear receptors," *Cell*, vol. 128, pp. 433-434, 2007.

[61] P. O. Danerud, "Brominated flame retardants as possible endocrine disruptors," *International Journal of Andrology*, vol. 31, no. 2, 2008.

[62] J. Legler and A. Brouwer, "Are brominated flame retardants endocrine disruptors?" *Environment International*, vol. 29, no. 6, pp. 879–885, 2003.

[63] J. H. Kamstra, E. Hruba, B. Blumberg et al., "Transcriptional and epigenetic mechanisms underlying enhanced in vitro adipocyte differentiation by the brominated flame retardant BDE-47," *Environmental Science & Technology*, vol. 48, no. 7, pp. 4110–4119, 2014.

[64] D. M. Walker and A. C. Gore, "Transgenerational neuroendocrine disruption of reproduction," *Nature Reviews Endocrinology*, vol. 7, no. 4, pp. 197–207, 2011.

[65] S. Rattan and J. A. Flaws, "The epigenetic impacts of endocrine disruptors on female reproduction across generations†," *Biology of Reproduction*, vol. 101, no. 3, pp. 635–644, 2019.

[66] E. F. Rissman and M. Adli, "Minireview: transgenerational epigenetic inheritance: focus on endocrine disrupting compounds," *Endocrinology*, vol. 155, no. 8, pp. 2770–2780, 2014.

[67] R. Newbold, R. B. Hanson, W. N. Jefferson, B. C. Bullock, J. Haseman, and J. A. McLachlan, "Increased tumors but uncompromised fertility in the female descendants of mice exposed developmentally to diethylstilbestrol," *Carcinogenesis*, vol. 19, no. 9, pp. 1655–1663, 1998.

[68] K. Inawaka, M. Kawabe, S. Takahashi et al., "Maternal exposure to anti-androgenic compounds, vinclozolin, flutamide and procymidone, has no effects on spermatogenesis and DNA methylation in male rats of subsequent generations," *Toxicology and Applied Pharmacology*, vol. 237, no. 2, pp. 178–187, 2009.

[69] A. Berger, A. Ziv-Gal, J. Cudiamat, W. Wang, C. Zhou, and J. A. Flaws, "The effects of in utero bisphenol A exposure on the ovaries in multiple generations of mice," *Reproductive Toxicology*, vol. 60, pp. 39–52, 2016.

[70] S. Santangeli, C. Consales, F. Pacchierotti, H. R. Habibi, and O. Carnevali, "Transgenerational effects of BPA on female reproduction," *Science of the Total Environment*, vol. 685, pp. 1294–1305, 2019.

[71] S. Salian, T. Doshi, and G. Vanage, "Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring," *Life Sciences*, vol. 85, no. 21-22, pp. 742–752, 2009.

[72] E. Brehm, S. Rattan, L. Gao, and J. A. Flaws, "Prenatal exposure to di(2-ethylhexyl) phthalate causes long-term transgenerational effects on female reproduction in mice," *Endocrinology*, vol. 159, no. 2, pp. 795–809, 2018.

[73] T. J. Doyle, J. L. Bowman, V. L. Windell, D. J. McLean, and K. H. Kim, "Transgenerational effects of di-(2-ethylhexyl) phthalate on testicular germ cell associations and spermatogonial stem cells in mice," *Biology of Reproduction*, vol. 88, pp. 112–9, 2013.

[74] M. Manikkam, R. Tracey, C. Guerrero-Bosagna, and M. K. Skinner, "Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations," *PLoS One*, vol. 7, 2012.

[75] W. R. Kelce, E. Monosson, M. P. Gamcsik, S. C. Laws, and L. E. Gray Jr., "Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites," *Toxicology and Applied Pharmacology*, vol. 126, no. 2, pp. 276–285, 1994.

[76] M. D. Anway, A. S. Cupp, M. Uzumcu, and M. K. Skinner, "Epigenetic transgenerational actions of endocrine disruptors and male fertility," *Science*, vol. 308, no. 5727, pp. 1466–1469, 2005.

[77] E. Nilsson, R. Klukovich, I. Sadler-Riggleman et al., "Environmental toxicant induced epigenetic transgenerational inheritance of ovarian pathology and granulosa cell epigenome and transcriptome alterations: ancestral origins of polycystic ovarian syndrome and primary ovarian insufficiency," *Epigenetics*, vol. 13, no. 8, pp. 875–895, 2018.

[78] J. A. Skinner, J. T. Wolstenholme, and E. F. Rissman, "Multi- and transgenerational consequences of bisphenol A on sexually dimorphic cell populations in mouse brain," *Endocrinology*, vol. 158, no. 1, pp. 21–30, 2016.

[79] J. T. Wolstenholme, J. A. Taylor, S. R. Shetty, M. Edwards, J. J. Connelly, and E. F. Rissman, "Gestational exposure to low dose bisphenol A alters social behavior in juvenile mice," *PLoS One*, vol. 6, no. 9, p. e25448, 2011.

[80] G. Schönfelder, W. Wittfoht, H. Hopp, C. E. Talsness, M. Paul, and I. Chahoud, "Parent bisphenol A accumulation in the human maternal-fetal-placental unit," *Environmental Health Perspectives*, vol. 110, no. 11, pp. a703–a707, 2002.

[81] M. Skinner and M. Anway, "Seminiferous cord formation and germ-cell programming: epigenetic transgenerational actions of endocrine disruptors," *Annals of the New York Academy of Sciences*, vol. 1061, pp. 18–32, 2005.

[82] P. Pocar, N. Fiandanese, A. Berrini, C. Secchi, and V. Borromeo, "Maternal exposure to di(2-ethylhexyl)phthalate (DEHP) promotes the transgenerational inheritance of adult-onset reproductive dysfunctions through the female germline in mice," *Toxicology and Applied Pharmacology*, vol. 322, pp. 113–121, 2017.

[83] K. M. Quinnies, T. J. Doyle, K. H. Kim, and E. F. Rissman, "Transgenerational effects of di-(2-ethylhexyl) phthalate (DEHP) on stress hormones and behavior," *Endocrinology*, vol. 156, no. 9, pp. 3077–3083, 2015.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 4802739, 7 pages
https://doi.org/10.1155/2020/4802739

Research Article

Selenium Supplementation, Body Mass Composition, and Leptin Levels in Patients with Obesity on a Balanced Mildly Hypocaloric Diet: A Pilot Study

Elisabetta Cavedon,[1] **Jacopo Manso**,[2] **Isabella Negro**,[3] **Simona Censi**,[2] **Roberto Serra**,[4] **Luca Busetto**,[4] **Roberto Vettor**,[4] **Mario Plebani**,[5] **Raffaele Pezzani**,[2,6] **Davide Nacamulli**,[2] **and Caterina Mian**[2]

[1]*Familial Cancer Clinic and Oncoendocrinology, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy*
[2]*Endocrinology Unit, Department of Medicine, University of Padua, Padua, Italy*
[3]*Internal Medicine, Alto Vicentino Hospital, Santorso (VI), Italy*
[4]*Internal Medicine 3, Department of Medicine, University of Padua, Padua, Italy*
[5]*Department of Laboratory Medicine, University of Padua, Padua, Italy*
[6]*AIROB, Associazione Italiana per La Ricerca Oncologica di Base, Padua, Italy*

Correspondence should be addressed to Jacopo Manso; jacopo.manso@gmail.com and Raffaele Pezzani; raffaele.pezzani@unipd.it

Received 8 January 2020; Revised 5 March 2020; Accepted 18 March 2020; Published 28 May 2020

Guest Editor: Rosanna Chianese

Background. Adequate levels of selenium (Se) have protective effects against several chronic diseases, such as obesity. The aim of this study was to assess the effect of Se supplementation in a selected group of patients with obesity. *Methods*. This randomized prospective study included 37 overweight/obese individuals aged 18–65 years, who adopted a slightly hypocaloric diet for 3 months. An intervention group received 240 μg/day of L-selenomethionine for 3 months; a control group received a placebo. Clinical and biochemical parameters, body composition measurements, and the Psychological General Well-Being Index (PGWBI) questionnaire were tested at the beginning and end of the treatment. *Results*. A comparison of the two groups showed a significant change in body composition, involving a decrease in body fat mass, between the baseline and the end of the follow-up, in the intervention group. Unlike the placebo group, the group given Se had a significant increase in lean body and muscle mass and a significant decrease in leptin levels after 3 months on diet. At the end of the follow-up, the group given Se scored higher on the PGWBI than those who did not. *Conclusion*. Se could reinforce the effects of diet for overweight and obesity. This work was registered in the ISRCTN registry with study ID ISRCTN6106073.

1. Introduction

Obesity is rapidly becoming one of the most worrying population health issues. It not only worsens an individual's quality of life, but also is associated with severe medical complications such as diabetes type 2, dyslipidemia, hypertension and other cardiovascular diseases, sleep apnea, various forms of cancer, pulmonary diseases, osteoarthritis, and a consequent increase in mortality/morbidity rates [1].

Leptin is an adiposity signal secreted by the adipocytes proportionally to the amount of body fat. In healthy individuals, it regulates the energy balance by increasing energy expenditure and reducing energy intake [2]. In diet-induced obesity, leptin levels rise due to leptin resistance, a condition deriving from activation of an inflammatory pathway, and from systemic oxidative stress, and involved in skeletal muscle atrophy, a disorder also linked to obesity and inflammation [3, 4]. In fact, obesity is associated with a state of chronic low-grade inflammation, and some studies have found blood concentrations of selenium (Se) inversely correlated with obesity, making Se deficiency a possible marker of adiposity [5–7]. Moreover, depression is also

described as an inflammatory condition, like obesity, with which it is often associated [8]. Lower Se blood concentrations correlate with a higher risk of depression and an Se-enriched diet seems to improve the psychoemotional state and mood of obese people [9, 10].

Se is a trace element involved in the proper functioning of the endocrine (and particularly, thyroid) and immune system and capable of modulating the body's inflammatory response due to the antioxidant action of selenoproteins [7]. Selenoproteins include glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases. Genetic knockout studies in mice have demonstrated that at least three selenoproteins are essential as deletion of thioredoxin reductase 1, thioredoxin reductase 2, or glutathione peroxidase 4 results in embryonic lethality [11–14]. In 1996, the World Health Organization (WHO) stated that daily intake up to 400 μg selenium could be considered safe [15]. However, a review by the United States Environmental Protection Agency stated that no adverse effect was seen in adults taking 853 μg/day of Se. Recommended dose of selenium varies in different countries, due to differences in geographical and racial characteristics, besides food habits of that population [16]. Moreover, optimal dose of Se intake in patients with metabolic diseases is difficult to estimate due to the limited amount of studies; however, most of these surveys stated that patients with metabolic disorders might need higher dose of Se than the healthy population (82.4–200 μg) [17].

Taken together, these findings suggest a promising role for Se supplementation in obesity, hence, this pilot study, the aim of which was to assess the effect on body weight and mood of high Se supplementation in a selected group of patients with obesity following a balanced, slightly hypocaloric diet as part of a single-center randomized controlled trial.

2. Materials and Methods

2.1. Patients and Study Design. This longitudinal, single-blind, randomized placebo-controlled clinical study involved 37 overweight and obese participants aged 18–65 years referred to our Endocrinology Unit for the purpose of losing weight (Figure 1). All participants had a body mass index (BMI) $\geq$25 kg/m^2. The following were reasons for exclusion: smoking habits, treatment with levothyroxine or any medication modifying thyroid function (e.g., corticosteroids, amiodarone, propranolol, and lithium), TSH levels outside the normal laboratory range, severe cardiopathy treated with antiarrhythmics or vasodilators, pregnancy or breastfeeding, previous or current malignancies, severe eating disorders, liver failure, pharmacological treatment for obesity, chronic inflammatory disease, individuals already on a low-calorie diet, or those who had followed a low-calorie diet in the previous 3 months or who had lost weight in recent months.

Participants were divided into two single-blind randomized groups: an intervention group ($n = 18$) took 240 μg/day of L-selenomethionine (S) in a soft gel formula divided into several doses for 3 months and a control group ($n = 19$) received placebo likewise delivered in a soft gel formulation (Figure 1). However, from the intervention group, 2 patients dropped out during the treatment. The reasons were a changed opinion regarding the study and the discovery of pregnancy, respectively.

After an interview with a clinical nutritionist, all participants adopted a balanced, slightly hypocaloric diet for 3 months. They were asked not to change their usual physical activity during the study period. Their clinical parameters and biochemical test findings were examined at the beginning and end of the treatment. Their body composition was measured and their psychological well-being was assessed, using the validated Psychological General Well-Being Index (PGWBI) questionnaire, at the beginning and end of the treatment.

The study was approved by the Research Ethics Committee of Padua University (protocol no. 3220/AO/14). All participants gave their written informed consent before enrolling for the study. This work was registered in the clinical trial ISRCTN registry with study ID ISRCTN6106073.

2.2. Clinical and Anthropometric Parameters. The clinical assessment included a general medical examination, recording arterial blood pressure, weight, and height. A participant's height was measured without shoes, with an approximation of 0.5 cm, and body weight was recorded without clothes, with an approximation of 0.1 kg. The BMI was calculated using the formula, weight [kg]/height^2 [m^2], and obesity was classified according to the World Health Organization [18].

2.3. Biochemical Tests. Serum leptin (expressed as μg/l) was measured at the beginning and at the end of the study using RIA (Radio Immuno Assay).

2.4. Body Composition. Body composition was assessed by bioelectrical impedance analysis (BIA) using the BIAVECTOR® (BODYGRAM-AKERN s.r.l. Bioresearch), recording values for lean mass, fat mass, and muscle mass at the beginning and end of the study for each participant.

2.5. Types of Diet. A tailored, slightly hypocaloric diet was given to all participants. The quantity of calories was calculated taking into account their basal metabolic rate obtained with the Harris-Benedict equation and reducing each participant's calorie requirement by 20–25%. As recommended by the European guidelines for obesity [19], the composition of the diet was balanced in the contribution of the main macronutrients according to the following criteria:

(i) Protein intake: 0.8–1.2 g/kg body weight reference/day

(ii) Carbohydrates: 55–65% of total kcal

(iii) Fat to supply the required amount of energy (30%): 10% monounsaturated, 10% polyunsaturated, and 10% saturated

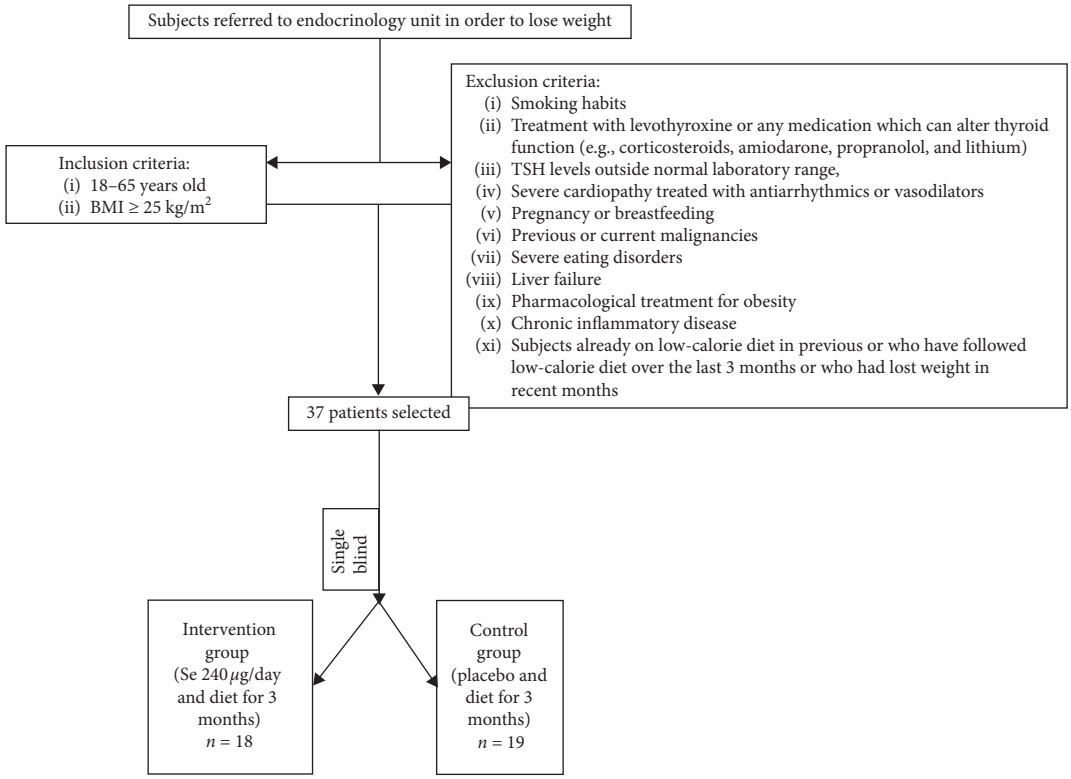


Figure 1: Study design with inclusion and exclusion criteria. Withdrawal due to discovery of pregnancy and changed opinion about the study.

2.6. Psychological General Well-Being Index (PGWBI). The PGWBI is based on a self-report questionnaire comprising 22 items and has proved to be capable of assessing respondents' stress levels [20, 21]. It has been validated and used in many countries in studies on large populations and specific groups. In 2000, the PGWBI was validated in a sample of 1,129 Italian citizens aged 15 years or more, and its normative values are available [20]. The questions cover six areas: anxiety, depressed mood, positive well-being, self-control, general health, and vitality. There are multiple-choice answers with scores ranging from 0 to 5. A total PGWBI score is obtained from the sum of all the items and ranges from 0 to 110. Higher scores indicate greater psychological well-being.

2.7. Statistical Analysis. For each continuous variable, a two-tailed Kolmogorov-Smirnov test was first performed to check the type of variable distribution. Then the t-test on independent samples, repeated-measures ANOVA, and the Mann–Whitney and Wilcoxon tests were used, as appropriate, to identify statistically significant differences. All statistical analyses were conducted using the *MedCalc* software bvba, Osted, Belgium (rel. 11.6.0). Values of $p < 0.05$ were considered statistically significant.

3. Results

No significant differences emerged between the two groups in terms of age, anthropometric measurements, or body composition at the beginning of the study. Table 1 shows participants' anthropometric characteristics at the baseline and at the end of the follow-up.

Statistical analysis showed that after 12 weeks, 14/35 (40%) participants had achieved a weight loss of at least 5% from their baseline body weight. Though the difference between the two groups was not statistically significant, the weight loss was more evident in the intervention group, in which 7/16 participants (44%) lost a considerable amount of weight, whereas this was only true of 7/19 (37%) in the placebo group.

There was a statistically significant difference, however, between the changes in body composition in the two groups. Only the intervention group showed a significant reduction in body fat mass from the baseline to the end of the follow-up ($p = 0.0002$). The results of ANOVA for repeated measures also showed that the placebo group's proportion of lean body mass did not change after 3 months on a hypocaloric diet, despite their significant body weight loss, whereas the proportion of lean body mass did change in the intervention group ($p = 0.01$). However, the difference in this parameter between the two groups did not reach statistical significance at the follow-up.

Considering only muscle mass changes, there were no differences in the placebo group after the hypocaloric diet, irrespective of their body weight, whereas participants in the intervention group showed a significant increase in muscle mass at the follow-up ($p = 0.02$).

Serum leptin levels dropped significantly in the intervention group after the diet ($p = 0.04$), while they remained the same in the placebo group (Figure 2).

Table 1: Anthropometric and biochemical characteristics of participants at the baseline and at the end of the study.

	Baseline			End of the study		
	Se-treated group ($n = 18$)	Placebo-treated group ($n = 19$)	p	Se-treated group ($n = 16$)	Placebo-treated group ($n = 19$)	p
Age (ys) ± SD	38 ± 10.9	42 ± 14.4	ns			
Gender			ns			
Male	5	6				
Female	13	13				
Weight (kg) ± SD	106.8 ± 31	114 ± 22.8	ns	103.1 ± 30.7	111.1 ± 21.9	ns
BMI (kg/m^2) ± SD	37.1 ± 8.9	41.8 ± 8.5	ns	36.9 ± 9.2	40.6 ± 7.7	ns
Fat mass (kg) ± SD	44.2 ± 3.8	47.1 ± 3.9	ns	34.5 ± 3.2	45.4 ± 3.8	0.03
Lean mass (kg) ± SD	62.5 ± 3.7	66.7 ± 2.9	ns	65.9 ± 3.4	66.9 ± 3.2	ns
Leptin levels ± SD	28.9 ± 16.3	28.6 ± 17.7	ns	23.9 ± 15.4	29.4 ± 16.9	0.01
PGWBI score ± SD	71 ± 17	79 ± 16.7	ns	82.3 ± 10	75.1 ± 21.2	0.003

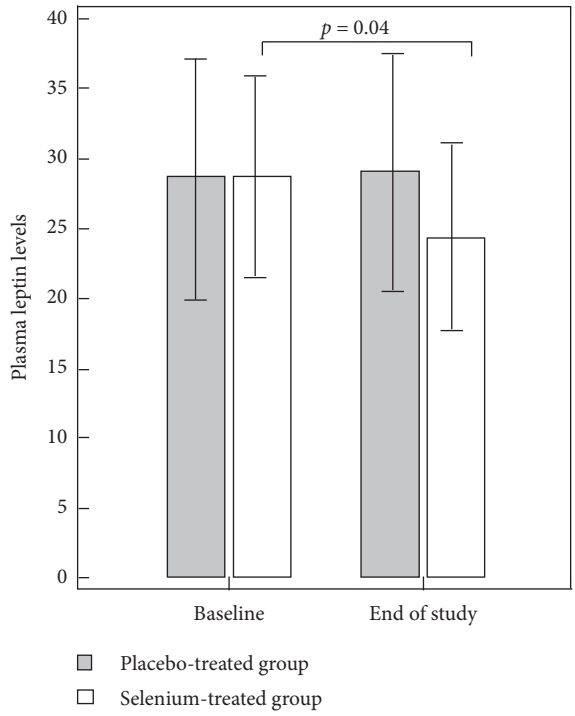


Figure 2: Comparison of plasma leptin levels (μg/l) at the baseline and after the isocaloric diet in the placebo-treated and selenium-treated groups.

Interestingly, participants who took Se scored higher on the PGWBI at the follow-up ($p = 0.003$), whereas there was no change in the placebo group's scores from the baseline to the follow-up. In addition, among all the participants who obtained a weight loss >5%, the change in their PGWBI scores was much more significant for those who had taken Se ($p = 0.004$).

4. Discussion

A balanced antioxidant status has a fundamental role in body homeostasis and has been linked to better health outcomes, particularly regarding some manifestations associated with obesity [4]. Selenium is an antioxidant micronutrient available in certain foodstuffs, such as fish, cereals, meat, and vegetables [22–24]. Adequate levels of Se have revealed a protective effect against various chronic diseases, like cancer prevention and cardiovascular protection [25]. Se supplementation exerts its beneficial action by increasing glutathione peroxidase activity and by interacting with inflammatory biomarkers [23, 24].

However, an unexpected result of the Nutritional Prevention of Cancer Trial in the USA was that participants treated with Se (200 μg/day) for an average follow-up of 7.7 years had an increased likelihood of developing type 2 diabetes comparing with a similar cohort of participants taking placebo [26]. However, some issues have to be taken into account: participants were elderly individuals (mean age 63.2 years old) with a self-reported diagnosis of type 2 diabetes and the increased risks for type 2 diabetes among Se-treated participants were no more evident when compared with patients within the top tertile of BMI (>26.76 kg/m^2), who usually have a dysregulated food habits, with inadequate consumptions of macro- and micronutrients.

Indeed, several studies have reported a positive correlation between serum Se concentrations and dietary Se intake and a negative correlation has been demonstrated between serum Se and BMI [26–28]. This relationship emerged, for instance, in a cross-sectional study conducted on 8- to 13-year-old schoolchildren; those with a BMI above the 85th percentile had a significantly lower dietary Se intake than normal-weight children, after adjusting for energy intake [28]. Data from the 1999 to 2004 US National Health and Nutrition Examination Survey (NHANES) also indicated that children at high risk of overweight were also at greater risk of dietary Se deficiency [29]. Finally, the CODING study, conducted on a large general adult population, clearly indicated that obesity and its severity were associated with a low dietary Se intake: every 1 μg/kg/day increase in dietary Se intake corresponded to a 3–6% decrease in the proportion of body fat mass [30].

The effect of dietary Se intake on body fat composition has been suggested by data emerging from animal interventional experiments. Wang et al. showed that body weight significantly decreased and the ratio of adipose tissue to body weight fell when rats were supplemented with high doses of

Se (200 μg/kg/day) [31]. This was due to a lipolytic effect in adipose tissue in parallel with a hepatic storage of free fatty acids. However, two small interventional studies in healthy humans generated contradictory findings [23, 32]. In a population of 54 normal-weight healthy volunteers, Hawkes and Keim found no effect of high-dose Se supplementation (297 μg/day) on body weight composition; in particular, their body fat status changed in the same way as in individuals treated with a low-Se diet (14 μg/day) [32]. More recently, a study conducted by Navas-Carretero et al. showed that the consumption of Se-enriched chickens by 11 individuals did not determine any significant weight loss vis-à-vis a group of 13 individuals who ate chickens not enriched with Se [23]. The authors concluded that this lack of effect of the Se-enriched diet was due to their population having sufficient dietary levels of Se at the baseline and to the low daily dose of Se (36.4 μg/day) added during the trial. In fact, there was no difference in participants' plasma Se levels before and after the supplementation period. The role of Se supplementation in lowering oxidative stress is actually only manifest in populations with endemic Se deficiency, as recently emphasized by several reports [33, 34].

To our knowledge, this is the first placebo-controlled trial of Se supplementation conducted in individuals with obesity. Our findings demonstrate that a high daily dose of Se was able to enhance the effect of a balanced, slightly hypocaloric diet by modifying body composition (reducing fat mass and increasing lean mass) in overweight/obese individuals from an area known to have a moderate Se deficiency, consistently with other reports [15, 22, 24, 35].

For now, the mechanisms behind the beneficial effect of dietary Se on body fat remain largely unclear. There are nonetheless some clues pointing to a link between Se and adipogenesis. Some previous studies applied Se in the differentiation of primary pig and rat preadipocytes and chicken embryonic fibroblasts, suggesting that Se may have a proadipogenic potential [30, 36, 37]. A recent study by Kim et al. showed that Se also inhibits adipogenesis by reducing mRNA expression of peroxisome proliferator-activated receptor-γ and fatty acid synthase. On the other hand, Se is capable of activating the expression of transforming growth factor-β [38]. But intraperitoneal injections of sodium selenite were able to reduce abdominal fat accumulation and adipocyte size in OLETF rats, stressing the antiadipogenic role of Se in vivo [30,39]. Moreover, Pitts et al. demonstrated that SelM, an endoplasmic reticulum-resident selenoprotein with antioxidant properties, was highly expressed in a hypothalamic area involved in energy metabolism and its deletion resulted in elevated serum leptin levels, increased adiposity, and hypothalamic leptin resistance [11]. On the contrary, in humans, rare mutations in the selenocysteine insertion sequence-binding protein 2, a protein required for selenocysteine incorporation into selenoproteins, were found to determine a multisystem selenoprotein deficiency disorder with paradoxical symptoms with enhanced insulin sensitivity and increased adiposity [40]. In summary, the evidence suggests that certain selenoproteins may act to promote adiposity and insulin resistance, while others may protect against it [11].

In our study, Se supplementation seemed to have a favorable impact on body mass remodeling, suggested by the association between dietary Se intake and a decrease in leptin levels in our Se-treated patients. It is well known that physiological leptin signaling is essential in maintaining body weight. Leptin resistance is a common characteristic of diet-induced obesity, in which anorectic responses to leptin are lower, and hyperleptinemia is a typical finding [4]. The mechanism that leads to leptin resistance is still unclear. Multiple factors, including inflammatory processes and oxidative stress and type of diet, may play a part. Se supplementation could therefore exert beneficial effects not only in reducing peripheral and central leptin resistance (through its antioxidant activity, by increasing selenoproteins activity, and by interacting with inflammatory biomarkers), but also may be via a direct effect on adipose tissue. However, in our survey, no assessments were performed to demonstrate improvement in antioxidant capacity in participants taking Se; for that reason we could only speculate that Selenium's biological activity as an antioxidant could underpin the effects mentioned above.

The glutathione antioxidant system might be implicated in the pathophysiology of mood disorders, too. Support for a role of the glutathione system in psychopathology comes from clinical trials involving treatment with N-acetyl cysteine (NAC), an antioxidant drug precursor of cysteine and glutathione: adjunctive NAC appears to be a safe treatment that has efficacy for schizophrenia [41]. In addition, Se might have protective role against neurodegenerative disease, like Parkinson's disease [42]. An optimal range of serum Se has been found to be associated with a lower risk of depressive symptoms too: higher Se levels correlated with lower scores on a geriatric depression scale and a lower risk of *de novo* major depressive disorders [5]. Prenatal Se supplementation seems to protect against postpartum depression as well [43]. In a double-blind US study involving 11 men confined to a metabolic unit for 120 days, low baseline dietary Se was associated with poor mood; nevertheless, intervention with a high Se diet of 356 μg/day for 99 days did not determine an improvement in mood; it is possible that the psychological effects of being confined may have counteracted any beneficial effects of the Se supplementation [44]. In a trial of 50 British participants, Se supplementation with 100 μg/day for five weeks determined improvement in depression, with a greater effect in individuals with poorer dietary Se intakes [45]. On the contrary, a larger randomised-controlled trial of elderly participants in the UK did not show such effect [46]. Nevertheless, recently Pasco et al. supported the hypothesis that lower dietary Se intakes increase the risk of *de novo* major depressive disorder [42].

In our survey, using a recognized quality of life assessment tool (the PGWBI), our study showed that Se supplementation might improve the mood of overweight/obese participants.

Our study suffers from several limitations that need to be mentioned. First, being a preliminary study, our failure to demonstrate any significantly greater weight loss in the Se-treated patients than in the placebo group may be due to the limited number of patients involved. A further, long-term Se

trial on a larger sample, with a sample size calculation, will be needed to confirm our promising preliminary data. In particular, it is fundamental to show that plasma leptin levels continue to drop as the body mass composition improves with Se supplementation. In addition, even if several studies reported a moderate-low Se deficiency status in our country, based on blood Se concentration, in our survey neither data about plasmatic Se levels at the baseline nor food habits, for a semiquantitative estimate of Se intake, were available to confirm Se deficiency in our patient cohorts. Lastly, none of our participants demonstrated any side effects following our brief course of Se supplementation, but it will be necessary to demonstrate that long-standing Se use has no adverse effects in patients with obesity.

In conclusion, our study seems to support the conviction that appropriate dietary Se supplementation may be useful in combating obesity. It could prove a simple and cost-effective intervention for individuals with overweight and obesity.

Data Availability

The data used to support the findings of this study are restricted by the Ethical Committee to protect patient privacy. Data are available from professor Caterina Mian for researchers who meet the criteria for access to confidential data.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to thank Frances Coburn for text editing and Adriano Tasinato and Diego Faggian for their excellent technical assistance.

References

[1] J. C. Halford, "Pharmacology of appetite suppression: implication for the treatment of obesity," *Current Drug Targets*, vol. 2, pp. 353–370, 2001.

[2] J. L. Chan, K. Heist, A. M. Depaoli, J. D. Veldhuis, and C. S. Mantzoros, "The role of falling leptin levels in the neuroendocrine and metabolic adaptation to short-term starvation in healthy men," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1409–1421, 2003.

[3] S. Leon-Cabrera, L. Solís-Lozano, K. Suárez-Álvarez, A. González-Chávez, L. Béjar, and G. Robles-Díaz, "Hyperleptinemia is associated with parameters of low-grade systemic inflammation and metabolic dysfunction in obese human beings," *Frontiers in Integrative Neuroscience*, vol. 7, pp. 1–7, 2013.

[4] N. Sáinz, J. Barrenetxe, M. J. Moreno-Aliaga, and J. A. Martínez, "Leptin resistance and diet-induced obesity: central and peripheral actions of leptin," *Metabolism*, vol. 64, no. 1, pp. 35–46, 2015.

[5] M. M. Donma and O. Donma, "Promising link between selenium and peroxisome proliferator activated receptor gamma in the treatment protocols of obesity as well as depression," *Medical Hypotheses*, vol. 89, pp. 79–83, 2016.

[6] A. Saedisomeolia, M. Allman-Farinelli, B. Hosseini, A. Saedisomeolia, and M. Allman-Farinelli, "Association between antioxidant intake/status and obesity : a systematic review of observational studies," *Biological Trace Element Research*, vol. 175, no. 2, pp. 287–297, 2016.

[7] G. J. Beckett and J. R. Arthur, "Selenium and endocrine systems," *Journal of Endocrinology*, vol. 184, no. 3, pp. 455–465, 2005.

[8] J. K. Soczynska, S. H. Kennedy, H. O. Woldeyohannes, C. Y. Yim, and R. S. Mcintyre, "Mood disorders and obesity : understanding inflammation as a pathophysiological nexus," *NeuroMolecular Medicine*, vol. 13, no. 2, pp. 93–116, 2011.

[9] M. A. Gosney, M. F. Hammond, A. Shenkin, and S. Allsup, "Effect of micronutrient supplementation on mood in nursing home residents," *Gerontology*, vol. 54, no. 5, pp. 292–299, 2008.

[10] S. Gao, Y. Jin, F. W. Unverzagt et al., "Selenium level and depressive symptoms in a rural elderly Chinese cohort," *BMC Psychiatry*, vol. 12, no. 1, 2012.

[11] M. W. Pitts, M. A. Reeves, A. C. Hashimoto et al., "Deletion of selenoprotein M leads to obesity without cognitive deficits," *Journal of Biological Chemistry*, vol. 288, no. 36, pp. 26121–26134, 2013.

[12] C. Jakupoglu, G. K. H. Przemeck, M. Schneider et al., "Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development," *Molecular and Cellular Biology*, vol. 25, no. 5, pp. 1980–1988, 2005.

[13] M. Conrad, C. Jakupoglu, S. G. Moreno et al., "Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function," *Molecular and Cellular Biology*, vol. 24, no. 21, pp. 9414–9423, 2004.

[14] L. J. Yant, Q. Ran, L. Rao et al., "The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults," *Free Radical Biology and Medicine*, vol. 34, no. 4, pp. 496–502, 2003.

[15] F. C. Gerald, "Review article Selenium in global food systems," *British Journal of Nutrition*, vol. 85, no. 5, pp. 517–547, 2019.

[16] M. Kieliszek and S. Błażejak, "Current knowledge on the importance of selenium in food for living organisms: a review," *Molecules*, vol. 21, no. 5, p. 609, 2016.

[17] N. Wang, H. Tan, S. Li, Y. Xu, W. Guo, and Y. Feng, "Review article supplementation of micronutrient selenium in metabolic diseases: its role as an antioxidant," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 7478523, 13 pages, 2017.

[18] WHO, *Obesity: Preventing and Managing the Global Epidemic. Report of a WHO Consultation*, Technical Report Series, World Health Organization, Geneva, Switzerland, 2000.

[19] V. Yumuk, C. Tsigos, M. Fried et al., "European guidelines for obesity management in adults," *Obesity Facts*, vol. 8, no. 6, pp. 402–424, 2015.

[20] E. Grossi and A. Compare, "Psychological general well-being index," *Encyclopedia of Quality of Life and Well-Being Research*pp. 5152–5156, Springer, Berlin, Germany, 2002nd edition, 2014.

[21] A. Carotenuto, A. M. Fasanaro, I. Molino, and F. Sibilio, "The psychological general well-being index (PGWBI) for assessing stress of seafarers on board merchant ships," *International Maritime Health*, vol. 64, no. 4, pp. 215–220, 2013.

[22] D. Nacamulli, C. Mian, D. Petricca et al., "Influence of physiological dietary selenium supplementation on the natural course of autoimmune thyroiditis," *Clinical Endocrinology*, vol. 73, pp. 535–539, 2010.

[23] S. Navas-Carretero, M. Cuervo, I. Abete, M. A. Zulet, and J. A. Martínez, "Frequent consumption of selenium-enriched chicken meat by adults causes weight loss and maintains their antioxidant status," *Biological Trace Element Research*, vol. 143, no. 1, pp. 8–19, 2011.

[24] M. P. Rayman, "The importance of selenium to human health," *The Lancet*, vol. 356, no. 9225, pp. 233–241, 2000.

[25] G. Flores-Mateo, A. Navas-Acien, R. Pastor-Barriuso, and E. Guallar, "NIH public access: selenium and coronary heart disease: a meta-analysis," *American Journal of Clinical Nutrition*, vol. 84, no. 4, pp. 762–773, 2006.

[26] S. Stranges, S. Sieri, M. Vinceti et al., "A prospective study of dietary selenium intake and risk of type 2 diabetes," *BMC Public Health*, vol. 21, no. 10, p. 564, 2010.

[27] M. Laclaustra, S. Stranges, A. Navas-Acien, J. M. Ordovas, and E. Guallar, "Serum selenium and serum lipids in US adults: national health and nutrition examination survey (NHANES) 2003-2004," *Atherosclerosis*, vol. 210, no. 2, pp. 643–648, 2011.

[28] M. Ortega, R. Rodríguez, I. Aparicio et al., "Young children with excess of weight show an impaired selenium status," *International Journal for Vitamin and Nutrition Research*, vol. 82, no. 2, pp. 121–129, 2012.

[29] Results from the 1999–2004 NHANES Dietary Intake in 2009.

[30] Y. Wang, X. Gao, P. Pedram et al., "Significant beneficial association of high dietary selenium intake with reduced body fat in the coding study," *Nutrients*, vol. 8, no. 1, p. 24, 2016.

[31] X. Wang, W. Zhang, H. Chen et al., "High selenium impairs hepatic insulin sensitivity through opposite regulation of ROS," *Toxicology Letters*, vol. 224, no. 1, pp. 16–23, 2014.

[32] W. C. Hawkes and N. L. Keim, "Human nutrition and metabolism metabolism in men," *Journal of Nutrition*, vol. 133, no. 11, pp. 3443–3448, 2003.

[33] C. D. Thomson, "Assessment of requirements for selenium and adequacy of selenium status : a review," *European Journal of Clinical Nutrition*, vol. 58, no. 3, pp. 391–402, 2004.

[34] C. D. Thomson, "Review article selenium and iodine intakes and status in New Zealand and Australia," *British Journal of Nutrition*, vol. 91, no. 5, pp. 661–672, 2018.

[35] D. Petricca, D. Nacamulli, C. Mian et al., "Effects of selenium supplementation on the natural course of autoimmune thyroiditis: a short review," *Journal of Endocrinological Investigation*, vol. 35, no. 4, pp. 419–424, 2012.

[36] K. Lee, D. B. Hausman, and R. G. Dean, "Expression of CCAAT/enhancer binding protein C/EBPα, β and δ in rat adipose stromal-vascular cells in vitro," *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, vol. 1450, no. 3, pp. 397–405, 1999.

[37] X. L. Chen, D. B. Hausman, and R. G. Dean, "Hormonal regulation of leptin mrna expression and preadipocyte recruitment and differentiation in porcine primary cultures of s-v cells," *Obesity Research*, vol. 6, no. 2, pp. 164–172, 1998.

[38] C. Y. Kim, G. N. Kim, J. L. Wiacek, C. Y. Chen, and K. H. Kim, "Selenate inhibits adipogenesis through induction of transforming growth factor-β1 (TGF-β1) signaling," *Biochemical and Biophysical Research Communications*, vol. 426, no. 4, pp. 551–557, 2012.

[39] J. E. Kim, S. I. Choi, H. R. Lee et al., "Selenium significantly inhibits adipocyte hypertrophy and abdominal fat accumulation in OLETF rats via induction of fatty acid β-oxidation," *Biological Trace Element Research*, vol. 150, no. 1–3, pp. 360–370, 2012.

[40] E. Schoenmakers, M. Agostini, C. Mitchell et al., "Mutations in the selenocysteine insertion sequence-binding protein 2 gene lead to a multisystem selenoprotein deficiency disorder in humans," *Journal of Clinical Investigation*, vol. 120, no. 12, pp. 4220–4235, 2010.

[41] W. Zheng, Q.-E. Zhang, D.-B. Cai et al., "N-acetylcysteine for major mental disorders: a systematic review and meta-analysis of randomized controlled trials," *Acta Psychiatrica Scandinavica*, vol. 137, no. 5, pp. 391–400, 2018.

[42] J. A. Pasco, F. N. Jacka, L. J. Williams et al., "Dietary selenium and major depression: a nested case-control study," *Complementary Therapies in Medicine*, vol. 20, no. 3, pp. 119–123, 2012.

[43] B. M. Y. Leung, B. J. Kaplan, C. J. Field et al., "Prenatal micronutrient supplementation and postpartum depressive symptoms in a pregnancy cohort," *BMC Pregnancy Childbirth*, vol. 13, no. 1, 2013.

[44] W. C. Hawkes and L. Hornbostel, "Effects of dietary selenium on mood in healthy men living in a metabolic research unit," *Biological. Psychiatry*, vol. 39, no. 2, pp. 121–128, 1996.

[45] D. Benton and R. Cook, "The impact of selenium supplementation on mood," *Biological Psychiatry*, vol. 29, no. 11, pp. 1092–1098, 1991.

[46] M. Rayman, A. Thompson, M. Warren-Perry et al., "Impact of selenium on mood and quality of life: a randomized, controlled trial," *Biological Psychiatry*, vol. 59, no. 2, pp. 147–154, 2006.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 7257913, 6 pages
https://doi.org/10.1155/2020/7257913

Research Article

Older Age Is Associated with Decreased Levels of VDR, CYP27B1, and CYP24A1 and Increased Levels of PTH in Human Parathyroid Glands

Yi Jiang,[1] Liyan Liao,[1] Jina Li,[2] Larry Wang,[3] and Zhongjian Xie[4]

[1]*Department of Pathology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China*
[2]*Department of Thoracic Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China*
[3]*Department of Pathology, Children's Hospital Los Angeles, University of Southern California, Los Angeles, CA 90027, USA*
[4]*Department of Metabolism and Endocrinology, Hunan Provincial Key Laboratory of Metabolic Bone Diseases, National Clinical Research Center for Metabolic Diseases, The Second Xiangya Hospital of Central South University, 139 Middle Renmin Road, Changsha 410011, Hunan, China*

Correspondence should be addressed to Zhongjian Xie; zhongjian.xie@csu.edu.cn

Received 17 June 2019; Revised 25 November 2019; Accepted 4 January 2020; Published 9 April 2020

Guest Editor: Renata S. Tavares

Parathyroid glands contain the vitamin D receptor (VDR) and 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1), which catalyze the production and degradation of 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], respectively. Previous studies have shown that the serum level of intact parathyroid hormone (iPTH) increases with age. We hypothesized that the expression of CYP27B1 or VDR in parathyroid glands decreases with age, which might account for the increased serum levels of iPTH due to decreased suppression of parathyroid hormone (PTH) secretion by 1,25$(OH)_2$D in older people. To test this hypothesis, we examined relative expression levels of VDR, CYP27B1, CYP24A1, and PTH in specimens from parathyroid glands unintentionally removed during thyroidectomy for 70 patients varying in age from 10 to 70 years. The results showed that there was an inverse correlation between age and VDR, CYP27B1, and CYP24A1 expression ($p < 0.05$). A significant positive correlation between PTH expression levels and age was also observed ($p < 0.05$). These data indicate that older age is associated with decreased levels of VDR, CYP27B1, and CYP24A1 and increased levels of PTH in human parathyroid glands.

1. Introduction

Vitamin D is hydroxylated by 25-hydroxylase in the liver to 25-hydroxyvitamin D [25(OH)D] that is subsequently hydroxylated by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the kidney to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. The latter is the active hormone that plays an important role in maintaining blood calcium and phosphorus levels and skeletal mineralization. 1,25$(OH)_2$D is catabolized to 1,24,25$(OH)_3$D by vitamin D-24-hydroxylase (CYP24A1) in target cells. It has been shown that 1,25$(OH)_2$D inhibits the synthesis and secretion of parathyroid hormone (PTH) and prevents proliferation of parathyroid glands [1, 2]. Serum levels of intact parathyroid hormone (iPTH) in the elderly population are reported to be higher than those in the younger population [3–5].

Parathyroid glands contain the vitamin D receptor (VDR) [6, 7], CYP27B1, and CYP24A1 [8]. Previous studies have shown that the expression of VDR decreases with age in cultured skeletal myocytes [9], human muscle tissue [10], rat intestine [11, 12], bone [11], and mammary glands [13]. However, it is unknown whether the expression of VDR, CYP27B1, or CYP24A1 is altered in parathyroid glands in older people. To address this question, we examined the expression levels, VDR, CYP27B1, CYP24A1, and PTH in human parathyroid glands in 70 patients undergoing thyroidectomy varying in age from 10 to 70 years.

2. Subjects and Methods

2.1. Parathyroid Specimens. Parathyroid specimens were collected from 70 parathyroid glands stored in the Department of Pathology of the Second Xiangya Hospital of Central South University. These 70 parathyroid glands were unintentionally removed during 6545 thyroidectomies performed between 2012 and 2016. Patients who underwent thyroidectomy had a diagnosis of the nodular goiter as a primary reason for surgery. All operations were performed at the Department of General Surgery, the Second Xiangya Hospital of Central South University. The study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University. Clinical records for all patients collected are detailed in Table 1. Sixteen specimens were from male patients, and 54 specimens were from female patients. The mean age was 41 ± 14 years. The serum calcium, phosphate, hepatorenal function, and intact PTH level were measured as the routine preoperative examination. All patients had normal levels of serum-corrected calcium, phosphate, eGFR, and iPTH, without calcium/phosphate metabolic diseases or renal dysfunction. Corrected calcium (cCa) was calculated by the formula: cCa (mmol/L) = tCa(mmol/L) + 0.025[40 − albumin(g/L) [14] where tCa represents total calcium.

2.2. Immunohistochemical Staining. The parathyroid specimens were fixed in formalin solution and embedded in paraffin blocks for routine immunohistochemical and hematoxylin-eosin staining. Paraffin-embedded 4-micrometer-thick specimens were dewaxed in turpentine and rehydrated through decreased concentrations of ethanol. Endogenous peroxidase activity was blocked by using 3% H_2O_2 in methanol for 15 min. The sections were incubated with trisodium citrate dihydrate liquid (0.125%, pH 6.0) for 15 min and then soaked with phosphate-buffered saline (PBS) liquid (pH 7.2–7.4) three times for 5 min. The sections were then preincubated with sheep serum for 10 min to block nonspecific antigen. The pretreated slides were incubated overnight at 4°C in a humidified chamber with antibodies to the VDR (1 : 100, cat#12550, rabbit monoclonal antibody from Cell Signaling Technology, Danvers, MA 01923, USA), CYP27B1 (1 : 100, cat#ABN182, rabbit polyclonal antibody from Upstate Technology, Lake Placid, NY, USA), CYP24A1 (1 : 100, cat#189322, goat polyclonal antibody from Abcam Inc., Cambridge, MA, USA), and PTH (ready to use, cat# MAB-0683, mouse monoclonal antibody from Maixin Biological Technology Development Co., Ltd., Fuzhou, China). After incubation with these antibodies, the slides were incubated at room temperature for 1 hour. After rinsing with PBS three times, the sections were incubated with secondary antibody anti-rabbit-HRP (KIT-9730, Maixin Biological Technology Development Co., Ltd.) or anti-goat-HRP (KIT-9719, Maixin Biological Technology Development Co., Ltd.) for 20 min, and the binding of peroxidase-conjugated secondary antibodies was detected with a DAB kit (Maixin Biological Technology Development Co., Ltd.). Hematoxylin was used for counter staining. Immunohistochemical staining of VDR in the human epidermis, CYP24A1 in the kidney, and CYP27B1 in liver was used as positive controls, respectively. PBS (pH 7.4) instead of the primary antibody was used as a negative control. Negative control slides were obtained in the corresponding tissue. Tissue for the positive control (epidermis, kidney, and liver) was preexisting and originally collected not for research purpose at the Department of Pathology of the Second Xiangya Hospital of Central South University. Use of the specimen in the present study was on the informed patient consent and approved by the Ethics Committee of the Second Xiangya Hospital of Central South University.

Table 1: Clinical features of 70 patients.

Demographic	Mean ± SD	Reference range
Age (year)	41 ± 14	
Calcium (mmol/L)	2.31 ± 0.12	2.11–2.52
Phosphorus (mmol/L)	1.04 ± 0.16	0.85–1.51
Creatinine (μmol/L)	53.31 ± 5.80	44–133
PTH (pmol/L)	4.32 ± 1.36	1.60–6.90

2.3. Image Analysis. The immunohistochemical staining was quantified by digital image procedures using ImageJ software (NIH, Bethesda, MD, USA) [15–20]. Tissue sections were viewed using bright-field illumination under a Leica DM LB2 upright light microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). The representative areas of the different sections were captured on a Leica DFC320 digital camera (Leica Microsystems Digital Imaging, Cambridge, UK). These images had a resolution of 2088 × 1550 pixels with RGB 24 true color format and were saved in uncompressed tagged-image file format (TIFF). The same range of illumination values were used to allow maximum reproducibility to avoid differences in the illumination. Captured images were converted to gray scale in ImageJ. Cells were manually marked out with a red pencil dot in Microsoft Paint, and the dots were then automatically identified and counted using ImageJ.

2.4. Statistical Analysis. The statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation. For the levels of VDR and CYP24A1 which comply with normal distribution, we used Pearson correlation analysis to evaluate their relationship with age. For the levels of CYP27A1 and PTH which did not comply with normal distribution, we used Spearman correlation analysis to evaluate their relationship with age. Results with p values < 0.05 were considered statistically significant, and all tests were two sided.

3. Results

To determine whether the expression of VDR, CYP27B1, CYP24A1, and PTH in human parathyroid glands is associated with age, we examined expression levels of VDR, CYP27B1, CYP24A1, or PTH-positive cells in parathyroid glands obtained from 70 patients with different ages using

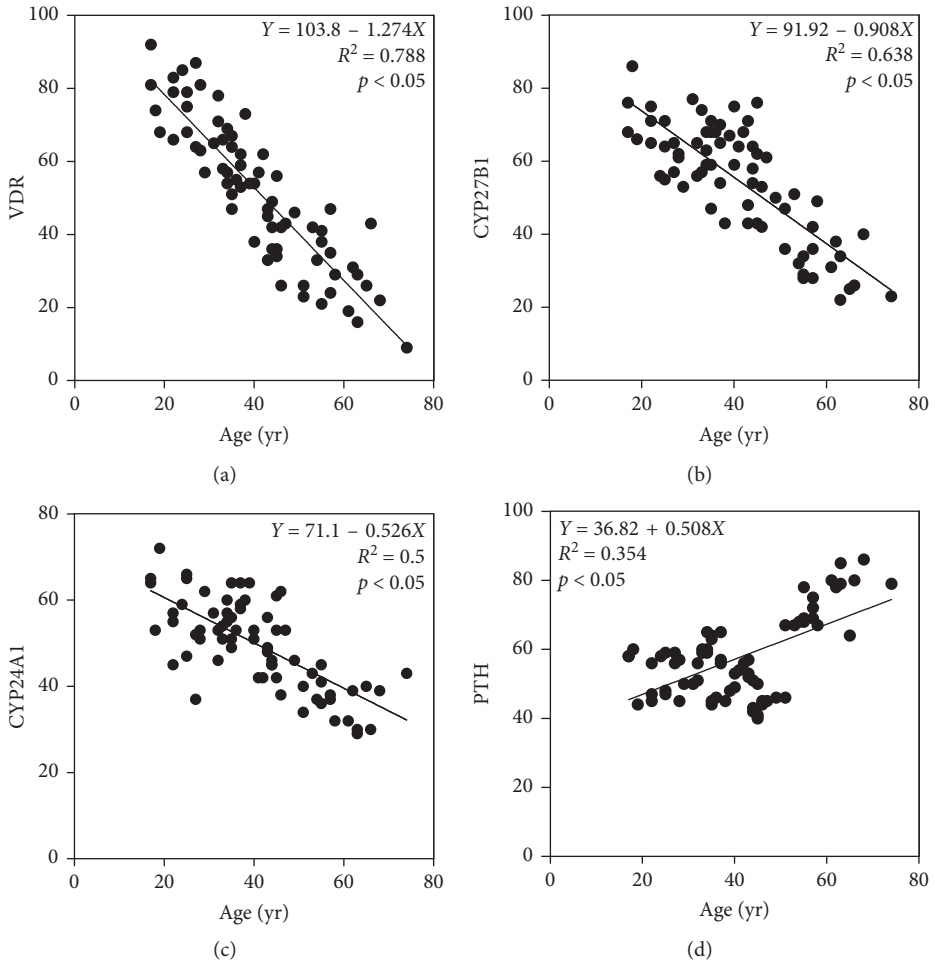


Figure 1: The association of (a) VDR, (b) CYP27B1, (c) CYP24A1, and (d) PTH expression levels in human parathydoid glands with age. Quantification of VDR, CYP27B1, CYP24A1, or PTH-positive cell rates in parathyroid glands of patients in different ages was performed by ImageJ software. Scatter plots of expression levels of VDR, CYP27B1, CYP24A1, or PTH versus age were shown.

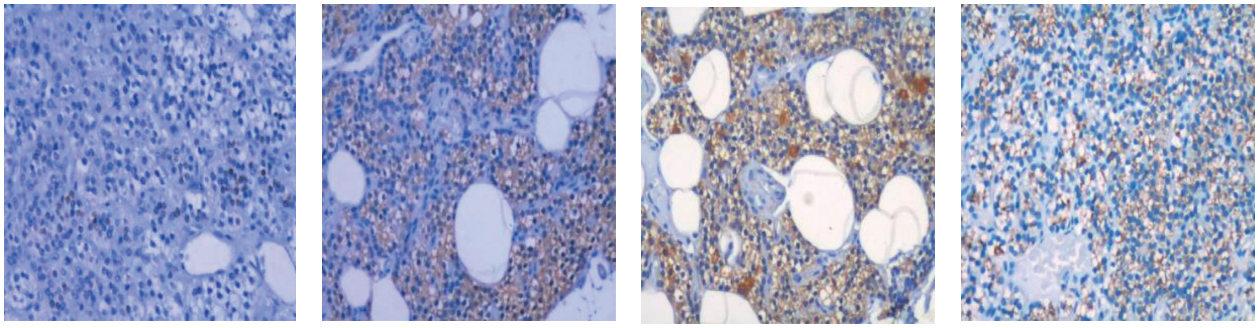
immunohistochemistry. Correlation studies between the expression levels of VDR, CYP27B1, CYP24A1, or PTH and age were performed. The results showed that age was inversely correlated with VDR ($r = -0.89$; $p < 0.0001$), CYP27B1($r = -0.74$; $p < 0.0001$), or CYP24A1 ($r = -0.71$; $p < 0.0001$) positive cell rates. A significant positively correlation between PTH and age was also seen ($r = 0.60$; $p < 0.0001$). The results are summarized in Figure 1. The expression of VDR was localized in the nucleus. The expression of CYP27B1 and CYP24A1 was located in the cytoplasm, and the expression of PTH was located in the cytoplasm and plasma membrane (Figure 2).

4. Discussion

In the present study, we used parathyroid tissue from patients with different ages to investigate the association of expression levels of VDR, CYP27B1, CYP24A1, and PTH in parathyroid glands. These patients had nodular thyroid goiters but had no pathological conditions affecting parathyroid glands. The results indicate that a decreased expression of VDR, CYP27B1, and CYP24A1 in parathyroid glands is associated with age. This is the first report to show an association of VDR, CYP27B1, and CYP24A1 with age in human parathyroid glands.

Vitamin D has been demonstrated to regulate cell proliferation, differentiation [21], apoptosis [22], angiogenesis [23], invasion, and metastasis [24]. Vitamin D exerts most of its biological activities by binding to VDR, which belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor [25].

VDR expression has been identified in many tissues and cell types [26], most notably monocytes and lymphocytes [27], glia [28], neurons [28], breast [29], and parathyroid [6, 7]. A number of studies have reported an association of VDR polymorphisms with Behçet's disease, diabetes, arthritis, autoimmune diseases, and hypertension [30–34]. Vitamin D levels may be affected by a number of factors including age, cultural behavior, latitude and season, and outdoor activity [35]. VDR has been found to be decreased with age in many tissues such as skeletal myocytes [9], human muscle tissue [10], rat intestine [11, 12], bone [11], and mammary glands [13]. The present data showed that



(a) (b) (c) (d)

Figure 2: Immunohistochemical staining of (a) VDR, (b) CYP27B1, (c) CYP24A1, and (d) PTH in human parathyroid glands. The specimens from seventy parathyroid glands were fixed in formalin solution and embedded in paraffin blocks for routine histological and immunohistochemical analysis using antibodies against VDR, CYP27B1, CYP24A1, or PTH. Immunohistochemical staining of VDR, CYP27B1, CYP24A1, or PTH is shown in brown, and the counterstaining is shown in blue in the representative section. VDR is located in the nucleus. CYP27B1 and CYP24A1 are located in the cytoplasm, and PTH is located in the cytoplasm and plasma membrane. PBS instead of the primary antibody was used as a negative control. Immunohistochemical staining of VDR in the epidermis, CYP24A1 in the kidney, and CYP27B1 in liver was used as positive controls, respectively (data not shown).

VDR expression levels in human parathyroid glands decreased with age.

The synthesis and degradation of $1,25(OH)_2D$ are regulated by CYP27B1 and CYP24A1, respectively. Immunocytochemical staining showed cytoplasmic staining with a microgranular pattern with antibodies against CYP27B1 and CYP24A1, which is consistent with their mitochondrial localization. We have also found a decreased expression of VDR, CYP27B1, and CYP24A1 with age in parathyroid glands. Decreased VDR expression levels in parathyroid glands in older people may lead to decreased responsiveness of parathyroid glands to $1,25(OH)_2D$, and decreased expression levels of CYP27B1 and CYP24A1 may lead to a decreased synthesis and degradation of $1,25(OH)_2D$.

The present result also showed increased levels of PTH in parathyroid glands in elderly adults compared with younger adults. These results are consistent with previous results of studies showed that iPTH levels increase with age in men and women and are about 30% higher in the elderly than in young subjects [36–39]. The increase in iPTH levels in the elderly has been attributed to declining renal function, declined calcium absorption efficiency, and declined 25(OH) D levels [3, 40]. However, other studies has been shown that iPTH levels significantly increase with age, independent of 25(OH)D levels, phosphate, and ionized calcium in the serum, and renal function [41].

Results from the present study which showed that VDR in parathyroid glands decreases with age may provide an explanation at least in part for the increase in iPTH in the elderly. Previous studies showing a decrease in the number of intestinal VDR with age leads to a decreased responsiveness of intestinal cells to $1,25(OH)_2D$ have demonstrated a similar pattern for VDR in different tissue from the elderly [42, 43].

The study has some limitations. A major limitation of the study is the relatively small number of samples included in the study. Another limitation is that the assessment of VDR, CYP27B1, and CYP24A1 levels in parathyroid glands were performed by a semiquantitative method.

In conclusion, a decreased expression of VDR, CYP27B1, and CYP24A1 and an increased expression of PTH in parathyroid glands are associated with age.

Abbreviations

VDR: Vitamin D receptor
$1,25(OH)_2D$: 1,25-dihydroxyvitamin D_3
PTH: Parathyroid hormone
iPTH: Intact parathyroid hormone
CYP27B1: 25-hydroxyvitamin D-1α-hydroxylase
CYP24A1: 25-hydroxyvitamin D-24-hydroxylase.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Yi Jiang and Jina Li are co-first authors.

Conflicts of Interest

None of the authors has conflicts of interest.

Authors' Contributions

Yi Jiang and Liyan Liao contributed to acquisition of specimens, performing experiments, and analysis and interpretation of data; Zhongjian Xie and Larry Wang oversaw design and provided critical feedback. All authors reviewed and revised the manuscript and approved the final manuscript as submitted and agreed to be accountable for all aspects of the work. Yi Jiang and Jina Li contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant numbers 81072219, 81272973, 81471055, and 81672646) and the Key Research and Development Program of Hunan Province (grant number 2019SK2253).

References

[1] J. Silver, T. Naveh-Many, H. Mayer, H. J. Schmelzer, and M. M. Popovtzer, "Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat," *Journal of Clinical Investigation*, vol. 78, no. 5, pp. 1296–1301, 1986.

[2] A. Canalejo, Y. Almadén, V. Torregrosa et al., "The in vitro effect of calcitriol on parathyroid cell proliferation and apoptosis," *Journal of the American Society of Nephrology: JASN*, vol. 11, no. 11, pp. 1865–1872, 2000.

[3] R. Vieth, Y. Ladak, and P. G. Walfish, "Age-related changes in the 25-hydroxyvitamin D versus parathyroid hormone relationship suggest a different reason why older adults require more vitamin D," *The Journal of Clinical Endocrinology & Metabolism*, vol. 88, no. 1, pp. 185–191, 2003.

[4] A. Valcour, F. Blocki, D. M. Hawkins, and S. D. Rao, "Effects of age and serum 25-OH-vitamin D on serum parathyroid hormone levels," *The Journal of Clinical Endocrinology & Metabolism*, vol. 97, no. 11, pp. 3989–3995, 2012.

[5] A. A. Portale, E. T. Lonergan, D. M. Tanney, and B. P. Halloran, "Aging alters calcium regulation of serum concentration of parathyroid hormone in healthy men," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 272, no. 1, pp. E139–E146, 1997.

[6] T. Uchiyama, N. Tatsumi, S. Kamejima et al., "Hypermethylation of the CaSR and VDR genes in the parathyroid glands in chronic kidney disease rats with high-phosphate diet," *Human Cell*, vol. 29, no. 4, pp. 155–161, 2016.

[7] S. Varshney, S. K. Bhadada, U. N. Saikia et al., "Simultaneous expression analysis of vitamin D receptor, calcium-sensing receptor, cyclin D1, and PTH in symptomatic primary hyperparathyroidism in Asian Indians," *European Journal of Endocrinology*, vol. 169, no. 1, pp. 109–116, 2013.

[8] U. Segersten, P. Björklund, P. Hellman, G. Åkerström, and G. Westin, "Potentiating effects of nonactive/active vitamin D analogues and ketoconazole in parathyroid cells," *Clinical Endocrinology*, vol. 66, no. 3, pp. 399–404, 2007.

[9] R. U. Simpson, G. A. Thomas, and A. J. Arnold, "Identification of 1,25-dihydroxyvitamin D3 receptors and activities in muscle," *The Journal of Biological Chemistry*, vol. 260, no. 260, pp. 8882–8891, 1985.

[10] H. Bischoff-Ferrari, M. Borchers, F. Gudat, U. Dürmüller, H. Stähelin, and W. Dick, "Vitamin D receptor expression in human muscle tissue decreases with age," *Journal of Bone and Mineral Research*, vol. 19, no. 2, pp. 265–269, 2004.

[11] R. L. Horst, J. P. Goff, and T. A. Reinhardt, "Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor," *Endocrinology*, vol. 126, no. 2, pp. 1053–1057, 1990.

[12] S. Takamoto, Y. Seino, B. Sacktor, and C. Tony Liang, "Effect of age on duodenal 1,25-dihydroxyvitamin D-3 receptors in Wistar rats," *Biochimica et Biophysica Acta (BBA)-General Subjects*, vol. 1034, no. 1, pp. 22–28, 1990.

[13] J. Welsh, L. N. Zinser, L. Mianecki-Morton et al., "Age-related changes in the epithelial and stromal compartments of the mammary gland in normocalcemic mice lacking the vitamin D3 receptor," *PLoS One*, vol. 6, no. 1, Article ID e16479, 2011.

[14] R. W. Pain, K. M. Rowland, P. J. Phillips, and B. M. Duncan, "Current "corrected" calcium concept challenged," *BMJ*, vol. 4, no. 5997, pp. 617–619, 1975.

[15] J. Schindelin, C. T. Rueden, M. C. Hiner et al., "The ImageJ ecosystem: an open platform for biomedical image analysis," *Molecular Reproduction and Development*, vol. 82, no. 7-8, pp. 518–529, 2015.

[16] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH Image to ImageJ: 25 years of image analysis," *Nature Methods*, vol. 9, no. 7, pp. 671–675, 2012.

[17] L. Handala, T. Fiore, Y. Rouille et al., "QuantIF: an ImageJ macro to automatically determine the percentage of infected cells after immunofluorescence," *Viruses*, vol. 11, no. 2, 2019.

[18] H. Mu, K. X. Lin, H. Zhao et al., "Identification of biomarkers for hepatocellular carcinoma by semiquantitative immunocytochemistry," *World Journal of Gastroenterology*, vol. 20, no. 19, pp. 5826–5638, 2014.

[19] F. Varghese, A. B. Bukhari, R. Malhotra et al., "IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples," *PLoS One*, vol. 9, no. 5, Article ID e96801, 2014.

[20] S. Chatterjee, R. Malhotra, F. Varghese et al., "Quantitative immunohistochemical analysis reveals association between sodium iodide symporter and estrogen receptor expression in breast cancer," *PLoS One*, vol. 8, no. 1, Article ID e54055, 2013.

[21] K. K. Deeb, D. L. Trump, and C. S. Johnson, "Vitamin D signalling pathways in cancer: potential for anticancer therapeutics," *Nature Reviews Cancer*, vol. 7, no. 9, pp. 684–700, 2007.

[22] A. S. Dusso, A. J. Brown, and E. Slatopolsky, "Vitamin D," *American Journal of Physiology-Renal Physiology*, vol. 289, no. 1, pp. F8–F28, 2005.

[23] D. J. Mantell, P. E. Owens, N. J. Bundred, E. B. Mawer, and A. E. Canfield, "1α,25-Dihydroxyvitamin D 3 inhibits angiogenesis in vitro and in vivo," *Circulation Research*, vol. 87, no. 3, pp. 214–220, 2000.

[24] C. M. Hansen, T. L. Frandsen, N. Brünner, and L. Binderup, "1α,25-Dihydroxyvitamin D3 inhibits the invasive potential of human breast cancer cellsin vitro," *Clinical & Experimental Metastasis*, vol. 12, no. 3, pp. 195–202, 1994.

[25] R. Evans, "The steroid and thyroid hormone receptor superfamily," *Science*, vol. 240, no. 4854, pp. 889–895, 1988.

[26] D. Zehnder, R. Bland, M. C. Williams et al., "Extrarenal expression of 25-hydroxyvitamin D3-1α-hydroxylase1," *The Journal of Clinical Endocrinology & Metabolism*, vol. 86, no. 2, pp. 888–894, 2001.

[27] D. Provvedini, C. Tsoukas, L. Deftos, and S. Manolagas, "1,25-Dihydroxyvitamin D3 receptors in human leukocytes," *Science*, vol. 221, no. 4616, pp. 1181–1183, 1983.

[28] K. Prüfer, T. D. Veenstra, G. F. Jirikowski, and R. Kumar, "Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat brain and spinal cord," *Journal of Chemical Neuroanatomy*, vol. 16, no. 2, pp. 135–145, 1999.

[29] N. Lopes, B. Sousa, D. Martins et al., "Alterations in vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1, and CYP24A1 expression in benign and malignant breast lesions," *BMC Cancer*, vol. 10, p. 483, 2010.

[30] A. G. Uitterlinden, Y. Fang, J. B. J. van Meurs, H. van Leeuwen, and H. A. P. Pols, "Vitamin D receptor gene polymorphisms in relation to vitamin D related disease

states," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 89-90, no. 1–5, pp. 187–193, 2004.

[31] S. Pilz and A. Tomaschitz, "Role of vitamin D in arterial hypertension," *Expert Review of Cardiovascular Therapy*, vol. 8, no. 11, pp. 1599–1608, 2010.

[32] G. Wuerzner, M. Burnier, and B. Waeber, "Should hypertensive patients take vitamin D?" *Current Hypertension Reports*, vol. 14, no. 4, pp. 318–323, 2012.

[33] N. Swapna, U. M. Vamsi, G. Usha, and T Padma, "Risk conferred by FokI polymorphism of vitamin D receptor (VDR) gene for essential hypertension," *Indian Journal of Human Genetics*, vol. 17, no. 17, pp. 201–206, 2011.

[34] M. A. Al-Barry, A. M. Albalawi, M. A. Sayf et al., "Sequence analysis of four vitamin D family genes (VDR, CYP24A1, CYP27B1 and CYP2R1) in Vogt-Koyanagi-Harada (VKH) patients: identification of a potentially pathogenic variant in CYP2R1," *BMC Ophthalmology*, vol. 16, no. 1, p. 172, 2016.

[35] A. Mithal, D. A. Wahl, J.-P. Bonjour et al., "Global vitamin D status and determinants of hypovitaminosis D," *Osteoporosis International*, vol. 20, no. 11, pp. 1807–1820, 2009.

[36] A. G. Need, P. D. O'Loughlin, H. A. Morris, M. Horowitz, and B. E. C. Nordin, "The effects of age and other variables on serum parathyroid hormone in postmenopausal women attending an osteoporosis center," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 4, pp. 1646–1649, 2004.

[37] A. Arabi, R. Baddoura, R. El-Rassi, and G. El-Hajj Fuleihan, "Age but not gender modulates the relationship between PTH and vitamin D," *Bone*, vol. 47, no. 2, pp. 408–412, 2010.

[38] J. M. Quesada, W. Coopmans, B. Ruiz et al., "Influence of vitamin D on parathyroid function in the elderly," *Journal of Clinical Endocrinology & Metabolism*, vol. 75, no. 2, pp. 494–501, 1992.

[39] S. T. Haden, E. M. Brown, S. Hurwitz, J. Scott, and G. E.-H. Fuleihan, "The effects of age and gender on parathyroid hormone dynamics," *Clinical Endocrinology*, vol. 52, no. 3, pp. 329–338, 2000.

[40] J.-C. Souberbielle, C. Cormier, C. Kindermans et al., "Vitamin D status and redefining serum parathyroid hormone reference range in the elderly," *The Journal of Clinical Endocrinology & Metabolism*, vol. 86, no. 7, pp. 3086–3090, 2001.

[41] S. J. Carrivick, J. P. Walsh, S. J. Brown, R. Wardrop, and N. C. Hadlow, "Brief report: does PTH increase with age, independent of 25-hydroxyvitamin D, phosphate, renal function, and ionized calcium?" *The Journal of Clinical Endocrinology & Metabolism*, vol. 100, no. 5, pp. 2131–2134, 2015.

[42] P. R. Ebeling, M. E. Sandgren, E. P. DiMagno, A. W. Lane, H. F. DeLuca, and B. L. Riggs, "Evidence of an age-related decrease in intestinal responsiveness to vitamin D: relationship between serum 1,25-dihydroxyvitamin D3 and intestinal vitamin D receptor concentrations in normal women," *The Journal of Clinical Endocrinology & Metabolism*, vol. 75, no. 1, pp. 176–182, 1992.

[43] P. V. Gonzalez and D. B. A. Russo, "Age-related changes in the response of intestinal cells to 1alpha,25(OH)2-vitamin D3," *Ageing Research Reviews*, vol. 12, no. 1, pp. 76–89, 2013.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 2750501, 8 pages
https://doi.org/10.1155/2020/2750501

Research Article

Fetal-Perinatal Exposure to Bisphenol-A Affects Quality of Spermatozoa in Adulthood Mouse

Teresa Chioccarelli,[1] **Francesco Manfrevola**,[1] **Marina Migliaccio**,[1] **Lucia Altucci**,[2] **Veronica Porreca**,[1] **Silvia Fasano**,[1] **and Gilda Cobellis**[1]

[1]*Department of Experimental Medicine, Sez. Bottazzi, Università degli Studi della Campania "L. Vanvitelli", Via Costantinopoli 16, 80138 Napoli, Italy*
[2]*Department of Precision Medicine, Università degli Studi della Campania "L. Vanvitelli", Via L. De Crecchio 7, 80138 Napoli, Italy*

Correspondence should be addressed to Gilda Cobellis; gilda.cobellis@unicampania.it

Received 2 December 2019; Revised 5 February 2020; Accepted 14 February 2020; Published 20 March 2020

Guest Editor: Matilde Cescon

Bisphenol-A (BPA) is considered an endocrine disruptor with estrogenic activity. It is described as an environment-polluting industrial chemical whose adverse effects on the male reproductive system depend on the period of exposure (i.e., fetal, prepubertal, or adult life). We exposed male mice to BPA during the fetal-perinatal period (from 10 days post *coitum* to 31 days post *partum*) and investigated the impact of this early-life exposure on gamete health in adulthood animals at 78 days of age. Both in control and BPA-exposed mice, viability and motility of spermatozoa, as well as sperm motility acquisition and chromatin condensation of spermatozoa, have been evaluated. Results reveal harmful effect of BPA on viability and motility of sperm cells as well as on chromatin condensation status during epididymal maturation of spermatozoa. In particular, BPA exposure interferes with biochemical mechanism useful to stabilize sperm chromatin condensation, as it interferes with oxidation of thiol groups associated to chromatin.

1. Introduction

During spermiogenesis, round spermatids undertake an extensive morphological transformation useful to form elongated mature spermatids (SPT) whose chromatin is mainly packaged by protamines more than histones (about 5% in mouse). Spermiation promotes detachment of mature SPT, i.e., spermatozoa (SPZ) from the nursing Sertoli cells. Simultaneously, a rhythmic contraction of peritubular myoid cells surrounding seminiferous tubules propels SPZ until the *caput* region of epididymis [1]. Sperm maturation occurs during epididymal transit from *caput*-to-*cauda*, as SPZ acquire their potential motility and further remodel some cellular compartments and structures [2, 3]. In eutherian mammals, including mouse and human, inter-/intra-protamine disulphide bridges formation occurs during the epididymal transit. This event further condenses chromatin, closing it in a tighter structure [4].

The neuroendocrine axis hypothalamus-hypophysis-gonad finely regulates the production of mature and quality gametes. It is known that androgens and, more recently, estrogens too have a key role in such modulation [5, 6]. Any interference with the neuroendocrine axis interferes with gamete quality.

Endocrine disruptors are environmental contaminants with anthropogenic origin, able to interfere with the endocrine system. Bisphenol-A (BPA) is described as endocrine disruptors, used in the manufacture of plastics and other products, largely present in the environment, with estrogenic activity [7]. Noteworthy, BPA shows estrogenic, antiestrogenic, and antiandrogenic activities; it binds estrogen receptor (ER) alpha (ERα; with agonistic and

antagonistic effects) or beta (ERβ; with agonistic effects) and interferes with thyroid hormone signalling [8–10]. Furthermore BPA may also act via membrane estrogen receptor by producing effects that are similar in potency to those of estradiol (E2) [11].

Consistently, clinical, epidemiological, and experimental studies show that the origins of some male reproductive tract disorders (i.e., cryptorchidism, low sperm count and quality, and infertility) can be traced back to the intrauterine period and show that fetal-perinatal BPA exposure, during the critical developmental/differentiation stages, may derange mechanisms controlling fertility in both animals and humans [12]. In fact, BPA is able to cross the placenta, transferring to the fetus [13] and later to the neonate, via milk [14], significantly increasing the risk of developing chronic diseases in the adult life [15]. However, BPA may accumulate in the embryo/fetal compartment after repeat maternal exposure, most likely as fetus is not able to efficiently metabolize BPA [16]. In this way, BPA differentially affects the male reproductive tract. The extent of its effects depends on the period of exposure (i.e., fetal, prepubertal, or adult life) [17]. In mouse, prenatal exposure to BPA adversely affects spermatogenesis in adulthood animals, with a reduction in the number of seminiferous tubules at stage VIII that predisposes sperm count decrease [18]. Accordingly, gestational exposure to BPA is reported to decrease the proportion of elongated SPT [19] as well as the number of SPZ [20]. In addition, men with different degrees of fertility, classified as slightly, moderately, and severely infertile men, reveal a negative association between seminal BPA levels (but not BPA plasma levels) and sperm concentration or total sperm count and morphology [21]. More recently, it has been reported that BPA induces breaks in DNA strands and generate reactive oxygen species in SPZ [22–24]. Other studies report a detrimental, dose-dependent effect of BPA on selected fertility-related proteins in SPZ as well as on function and fertilization ability of sperm cells [25]. Although adverse effect of BPA on semen quality is consistent, there is insufficient evidence to draw conclusions about how BPA interferes with sperm health. Therefore, we performed an *in vivo* study to evaluate the possible effects of BPA exposure on SPZ. Specifically, we evaluated the impact of the fetal-perinatal exposure on gamete health in adulthood animals.

2. Materials and Methods

2.1. Experimental Design and BPA Exposure. The experimental design has been structured to avoid undesired environmental contamination of BPA. Accordingly, standard polypropylene cages (Tecniplast S.p.A., Varese, Italy), corncob bedding (Envigo srl, Udine, Italy), and glass bottles (Zooplus AG, Monaco di Baviera, Germany) were used [26].

Mice strain (CD1, Charles River), diet (Envigo srl, Udine, Italy), and BPA concentration (10 μg/mL, Sigma-Aldrich, Milano, Italy), as well as route (drinking water), time (from 10 days *post coitum* (*dpc*) to 31 days *post partum* (*dpp*)) and period (foetal/perinatal) of exposure were chosen in agreement with effectiveness of the experimental plan reported by Miyawaki and coworkers [27]. In particular, we chose 10 μg/mL as *in vivo* concentration according to "safe" dose of BPA for human by U.S. European Protection Agency.

Male mice were exposed to drinking water containing ethanol alone (0.2% as vehicle; $n = 8$, unexposed/control group; CTRL) or containing BPA (10 μg/mL BPA dissolved in 0.2% ethanol; $n = 12$, exposed group), via pregnant/nursing mothers (from 10 *dpc* to 21 *dpp*) o via direct access to water (from 21-to-31 *dpp*).

After weaning, each male litter ($n = 5$ litters/CTRL group; $n = 5$ litters/BPA-exposed group) was housed in a single cage, and some physiological parameters (e.g., weight, food intake, etc.) were constantly monitored from 21-to-78 *dpp*, in both experimental groups [28]. The animals were sacrificed at 78 *dpp* and subjected to tissue collection. To note, before sacrifice, food was removed from the cage at 5:00 pm and animals were killed the day after, between 9:00 and 11:30 am, under ether anaesthesia by cervical dislocation. Epididymis were accurately removed, and the *caput* and *cauda* region were properly processed for SPZ collection from *caput* (*caput* SPZ) and *cauda* (*cauda* SPZ), separately.

Experiments were approved by the Italian Ministry of Education and the Italian Ministry of Health, with authorization *n*° 941/2016-PR issued on 10.10.2016.

Procedures involving animal care were carried out in accordance with National Research Council's publication *Guide for Care and Use of Laboratory Animals* (National Institutes of Health Guide).

2.2. Spermatozoa Collection. *Caput* and *cauda* SPZ ($n = 5$ for CTRL group; $n = 5$ for BPA group) were collected from the relative epididymal segment. In particular, *caput* and *cauda* of epididymis were separately immersed in phosphate buffer saline (PBS, pH 7.6) and cut into few pieces to let the SPZ flow out from the ducts. SPZ samples were then filtered and immediately used ($n = 5$ for the experimental group) to analyse the number of live, motile, and total cells. Aliquots of SPZ samples were fixed ($n = 3$ for experimental group) and later used to evaluate sperm chromatin parameters such as condensation and disulfide bound formation.

2.3. Analysis of Live and Motile Spermatozoa. We used CTRL ($n = 4$) and BPA-exposed ($n = 4$) mice to analyze the number of live and motile SPZ from *caput* and *cauda* of epididymis. The number of live and motile SPZ was evaluated under a light microscope (magnification 20X) using a haemocytometer (Burker Chamber). This procedure was validated using double-blind test. Live SPZ were evaluated using the viable dye tripan blue and plotted as percentage of live/total SPZ. Motile SPZ were count and plotted as percentage of motile/live SPZ.

2.4. Acridine Orange (AO) Staining Analysis. The fluorochrome AO intercalates into double strand DNA (native DNA) as a monomer and fluoresces green. Conversely, when

it binds to single strand DNA (denatured or single strand DNA) as an aggregate, a red fluorescence is observed. Noteworthy, DNA is vulnerable to denaturation under acid conditions [29, 30]. This metachromatic shift from green (FL1-H) to red (FL3-H) has been used to measure chromatin quality indices of SPZ under acid conditions [31, 32].

Using cytofluorimetry analyses, we evaluated the percentage of SPZ with high DNA stainability (i.e., HDS) as well as thiol/disulphide status (i.e., TDS) in sperm samples collected from *caput* and *cauda* region of epididymis. Values were considered as spermatic indices of uncondensed chromatin (i.e., HDS, calculated as intensely green (FL1-H $> 10^5$) fluorescing DNA/total fluorescing DNA (FL1-H $> 10^3$ + FL3-H $> 10^3$)) and thiol groups oxidation (i.e., TDS, calculated as red fluorescing [FL3-H $> 10^3$]/green fluorescing (FL1-H $> 10^3$) DNA), respectively [5, 29, 31, 32].

Aliquots of SPZ (1×10^6/100 μL) collected from *caput* or *cauda* epididymis were suspended in 1 ml of ice-cold PBS (pH 7.4) buffer and centrifuged at 600g for 5 minutes. The pellet was resuspended in ice-cold TNE (0.01 M Tris-HCl, 0.15 M NaCl and 1 mM EDTA, pH 7.4) buffer and again centrifuged at 600g for 5 minutes. The pellet was then resuspended in ice-cold TNE-10% glycerol buffer (200 μL) and immediately fixed in ethanol (70% v/v) at 4°C for 24 h. Cytofluorimetry analysis was simultaneously carried out on *caput* and *cauda* SPZ from CTRL vs. BPA-exposed mice. The samples were treated for 30 seconds with 400 μL of a solution of 0.1% Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.2. After 30 seconds 1.2 mL of staining buffer (6 μg/mL AO, 37 mM citric acid, 126 mM Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, pH 6.0) was admixed to the test tube and analyzed by flow cytometry. After excitation by a 488 nm wavelength light source, AO bound to a double-stranded DNA fluoresces green (515–530 nm) and AO bound to a single-stranded DNA fluoresces red (630 nm or greater). A minimum of 10,000 cells were analyzed by fluorescent activated cell sorting (FACSCalibur, BD Bio-Science, Milan, Italy).

2.5. Incubation of SPZ with BPA by In Vitro Experiment. Spermatozoa collected from *caput* epididymis of adult mice ($n = 3$) were treated with vehicle or with two different doses of BPA, 2 ng/mL and 20 ng/mL, corresponding to 0.01 μM and 0.1 μM, respectively. These doses are below the acceptable human daily exposure levels [33]. Each treatment lasted 15 min and was carried out at room temperature. Afterward, the number of total, live, and motile SPZ was evaluated as described above.

To preserve control quality and avoid any effect due to the vehicle, all experimental groups received ethanol (0.01%), being BPA dissolved in ethanol.

2.6. Statistical Analysis and Data Presentation. Student's t-test and Duncan's test (for multigroup comparison) were carried out to evaluate the significance of differences. Data are expressed as the mean ± S.E.M.

3. Results

3.1. BPA Effects on Viability of Caput Spermatozoa. Spermatozoa collected from *caput* epididymis of CTRL and BPA-exposed mice have been used to evaluate live SPZ. In particular, the number of live and total cells was analysed, and data were reported as percentage of live/total SPZ.

The percentage of live SPZ (Figure 1(a)) from *caput* epididymis was significantly higher ($p < 0.01$) in the CTRL group as compared with BPA-exposed animals.

3.2. BPA Effects on Sperm Motility Acquisition. To study the interference of BPA on epididymal sperm maturation and, in particular, on sperm motility acquisition during the epididymal transit, from *caput*-to-*cauda*, we analyzed the percentage of motile SPZ from *cauda* and *caput* epididymis of CTRL and BPA-exposed mice (Figure 1(b)). In particular, the number of live, motile, and total cells was analysed, and data were reported as percentage of motile/live SPZ.

In CTRL and BPA-exposed mice, the percentage of motile SPZ significantly increased from *caput* to *cauda* ($p < 0.01$ or $p < 0.05$). The percentage of motile SPZ from *caput* epididymis was significantly higher ($p < 0.05$) in the CTRL group as compared with the BPA-exposed animals, while comparable percentage values of motile SPZ were observed in *cauda* epididymis of CTRL and BPA-exposed group.

3.3. BPA Effects on Caput SPZ by In Vitro Incubation. To investigate the harmful BPA effects on viability and motility of *caput* SPZ, we planned *in vitro* experiments and specifically incubated *caput* SPZ with vehicle (CTRL) or with two different doses of BPA (2 and 20 ng/mL). The number of live, motile, and total sperm cells was evaluated, and data were reported as the percentage of live/total SPZ or motile/live SPZ.

Results show that viability of SPZ was unresponsive to the direct BPA exposure (Figure 2(a)). Indeed, both doses (2 and 20 ng/mL) were not able to decrease the number of live SPZ. Interestingly, the higher dose of BPA (20 ng/mL) significantly decreased the percentage of motile SPZ as compared to the CTRL group ($p < 0.05$), while no significant difference was observed at the lower doses (2 ng/mL) (Figure 2(b)).

3.4. Effects of BPA on Chromatin Condensation of Caput and Cauda Spermatozoa. Sperm samples from *caput* and *cauda* epididymis of CTRL and BPA-exposed mice have been stained with AO dye in acid conditions and analyzed by flow cytometry. HDS and TDS have been evaluated and used as spermatic indices of uncondensed chromatin and thiol-disulphide status, respectively.

Figure 3(a) shows histograms of green-stained (FL1-H) and red-stained (FL3-H) *caput* and *cauda* SPZ from CTRL and BPA-exposed mice, in the gated areas (M1 and M2, respctively), while the Figures 3(b) and 3(c) show the relative HDS and TDS values, respectively.

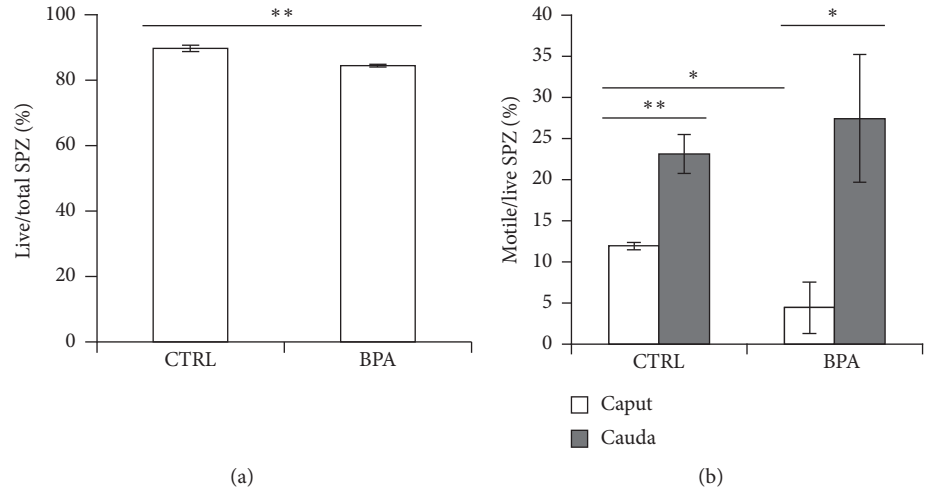


Figure 1: Viability (a) of spermatozoa (SPZ) collected from caput epididymis of mice exposed to vehicle (control, CTRL) or Bisphenol-A (BPA). (b) Motility of SPZ collected from caput and cauda epididymis of mice exposed to vehicle (CTRL) or BPA. Data are reported as percentage of motile/live SPZ ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$.

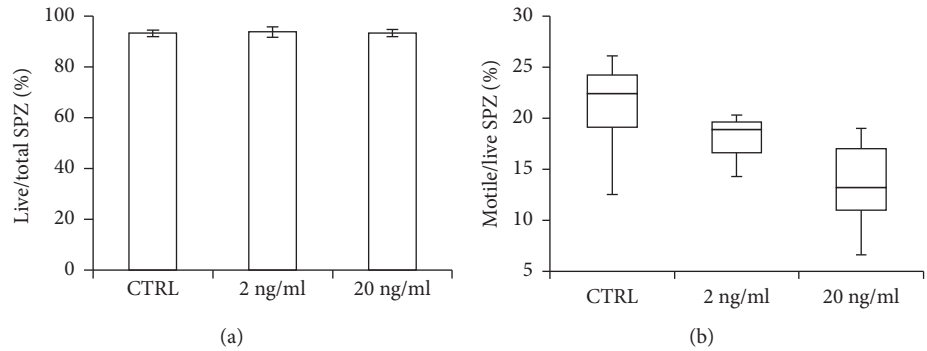


Figure 2: Viability (a) and motility (b) of spermatozoa (SPZ) collected from caput epididymis of adult mice incubated with vehicle (control, CTRL) or with two different doses of Bisphenol-A (BPA) (2 and 20 ng/ml). Data are reported as percentage of live/total SPZ (a) and motile/live SPZ (b) ±S.E.M. $^{*}p < 0.05$.

In CTRL mice, HDS values showed no significant difference, CTRL vs. BPA, while, in BPA-exposed mice significantly increased from *caput* to *cauda* epididymis ($p < 0.05$). However, in *cauda* epididymis, HDS values were higher in BPA-exposed mice than in the CTRL group ($p < 0.05$).

In CTRL mice, TDS values significantly decreased from *caput* to *cauda* epididymis ($p < 0.05$) while, in BPA-exposed mice, no significant difference was detected from the *caput* to *cauda* region.

4. Discussion

Male mice exposed to BPA during the fetal-perinatal period have been sacrificed at 78 *dpp*. Spermatozoa collected from *caput* and *cauda* epididymis have been morphologically and biochemically analyzed. The aim was to evaluate the impact of the fetal-perinatal exposure on gamete health in adulthood animals. Phenotypic parameters and chromatin features have been considered to qualitatively study the gamete as the result of events related to spermatogenesis and epididymal maturation. Phenotypic parameters of SPZ, such as viability and motility, have been evaluated by analysis of *caput* SPZ, while epididymal sperm maturation processes, such as motility acquisition and chromatin condensation, have been evaluated by analysis of *caput* and *cauda* SPZ.

Results demonstrate that BPA exposure decreased the number of live or motile SPZ in *caput* epididymis revealing adverse effects of BPA on viability and motility of sperm cells. Interestingly, BPA disturbed spermatogenesis as seminiferous tubules of BPA-exposed mice shown exfoliated germ cells in tubular lumen, particularly elongating SPT and SPZ (data not shown). However, despite in *caput* epididymis BPA decreased the number of motile SPZ, no effect was observed in the *cauda* region. Number of motile SPZ increased from *caput* to *cauda*, both in CTRL and BPA-exposed mice, with comparable values in *cauda*, CTRL vs. BPA, revealing that SPZ efficiently acquired their potential to move during the epididymal transit. This occurred independently by exposure, demonstrating that BPA did not interfere with sperm motility acquisition mechanism while it specifically counteracted sperm motility in *caput* epididymis.

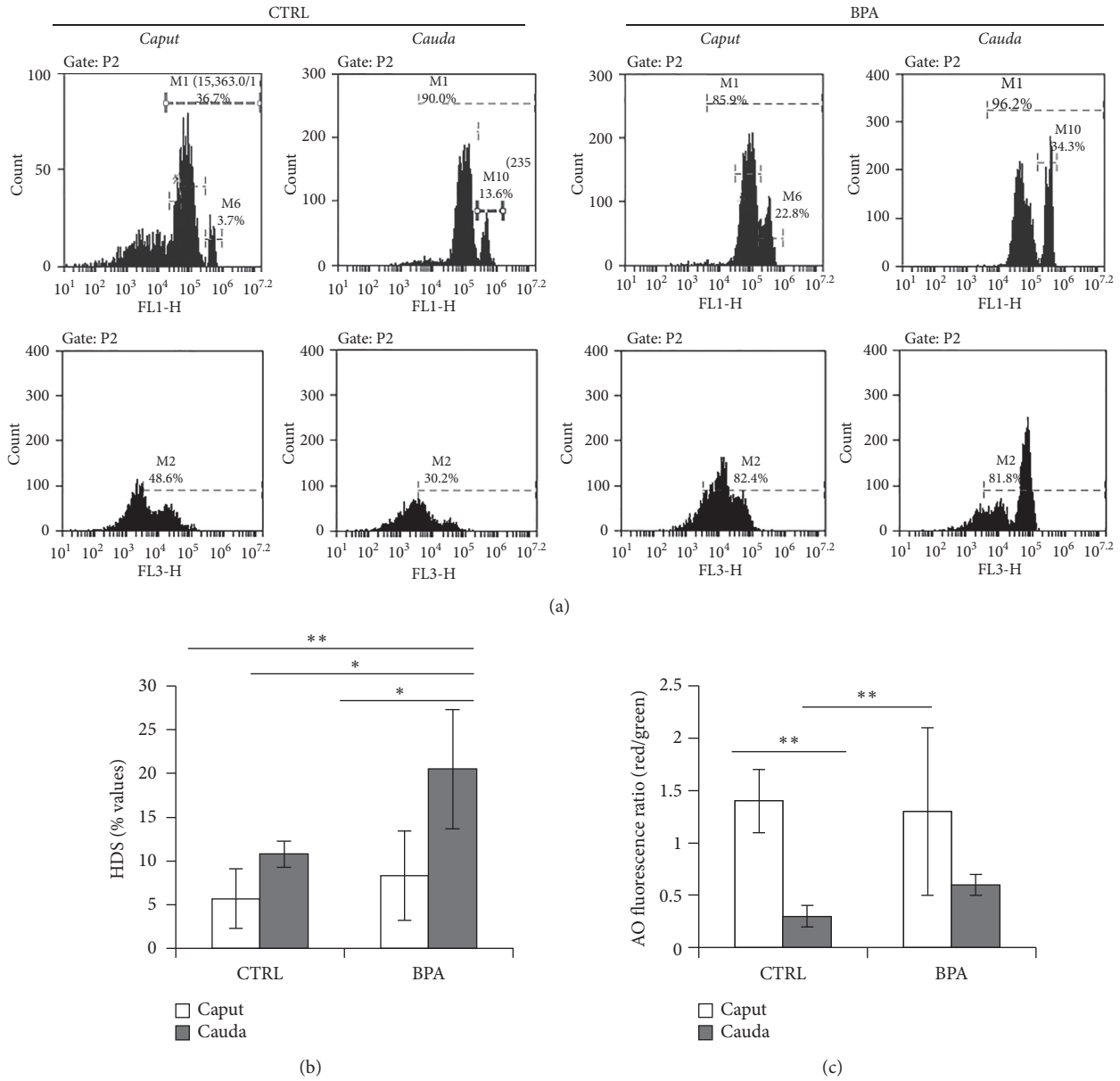


Figure 3: Flow cytometry analysis of caput and cauda spermatozoa (SPZ) from mice exposed to vehicle (control, CTRL) or Bisphenol-A (BPA). (a) Representative histograms of Acridine orange (AO) stained sperm in M1- and M2-gated areas. Intensely green (FL1-H > 10^5), green (FL1-H > 10^3), red (FL3-H > 10^3), and total (green + red) fluorescencing DNA were used to analyze (b) high DNA stainability (HDS) and (c) thiol/disulphide status (TDS) values. Graphs were representative of three sperm samples relative to separate animals. Data were expressed as the mean ± S.E.M. $^*p < 0.05$ and $^{**}p < 0.01$.

To investigate the harmful effect of BPA on viability and motility of SPZ transiting in *caput* epididymis, using an *in vitro* incubation system, we directly exposed *caput* SPZ to vehicle or BPA (2 and 20 ng/ml) and analyzed the number of live or motile SPZ. No effect was observed on live sperm number, while a negative dose-dependent BPA-effect was noticeable on number of motile SPZ. This revealed that BPA was able to counteract sperm motility by direct action on SPZ, while it was not effective on viability. This observation, if translated in our *in vivo* model and, in particular, in *caput* epididymis of BPA-exposed mice, suggests that the higher number of dead SPZ was probably referable to harmful BPA-effect on germ cells during spermatogenesis. On the contrary, the lower number of motile SPZ was realistically ascribable to a local accumulation and action of BPA in the epididymal *caput* region. In agreement, the differential accumulation of endocrine disruptors, including BPA, has been reported in different animal tissues [34] including the visceral fat mass, which in our exposure experimental model preferably accumulates BPA [35]. Interestingly, the gestational BPA exposure adversely affects spermatogenesis in adulthood mice. Such exposure interferes with development of elongated SPT, as BPA likely acts as estrogenic chemical [19]. Indeed, estrogens lead to degeneration of elongated SPT in young rats *in vivo* exposed to high doses [36], while

in mouse they modulate chromatin remodeling of SPT during spermiogenesis [37, 38]. In particular, 17β-Estradiol is reported to facilitate histone-protamine exchange in mouse SPT by promoting histone displacement [5]. Such event predisposes chromatin condensation extent of *caput* SPZ as well as predisposes *caput* SPZ to preserve chromatin condensation status during the epididymal transit by inter-/intra-protamine disulphide bridge formation [4].

With this in mind using a biochemical approach, based on AO fluorescent dye under acid conditions, we analyzed the percentage of SPZ with high DNA stainability (i.e., HDS) as well as thiol/disulphide status (i.e., TDS) in sperm samples collected from *caput* and *cauda* of epididymis of CTRL and BPA-exposed mice. Values were considered as spermatic indices of uncondensed chromatin (i.e., HDS) and thiol groups oxidation (i.e., disulphide bridges formation), respectively.

Results revealed scanty HDS values in *caput* epididymis, both in CTRL and BPA-exposed mice, with no significant difference, CTRL vs. BPA, demonstrating no effective BPA interference on spermatogenetic mechanisms that predispose chromatin condensation extent in *caput* SPZ. However, in *cauda* epididymis, the percentage of SPZ with uncondensed chromatin (i.e., HDS) was higher in BPA-exposed mice than in the CTRL group, suggestive harmful BPA effect on *cauda* SPZ. As expected, no significant differences was observed in the CTRL group when we analyzed HDS values in *caput* and *cauda* epididymis, confirming that SPZ preserve their chromatin condensation status during the epididymal transit. Conversely, in BPA-exposed mice, the HDS values increased from *caput*-to-*cauda*. This revealed higher susceptibility of sperm chromatin to swelling in *cauda* compared to *caput* epididymis. Noteworthy, during the epididymal transit of mouse sperm, inter-/intra-protamine thiol group oxidation strongly stabilizes sperm chromatin condensation status [4, 39, 40]. With this in mind, we hypothesized that BPA exposure interfered with epididymal sperm chromatin maturation by affecting disulphide bridge formation. In agreement, the thiol/disulphide ratio (i.e., TDS value) decreased in CTRL mice from *caput*-to-*cauda* SPZ, while in BPA-exposed mice, it was stably elevated in SPZ transiting from the *caput*-to-*cauda* region of epididymis, demonstrating that BPA-exposure counteracted the oxidation of the thiol groups associated to sperm chromatin.

In conclusion, our results show that mice exposed to BPA during the fetal-perinatal period produce poorly healthy gamete in adulthood. We show harmful effect of BPA on viability and motility of sperm cells as well as on sperm chromatin maturation extent. In particular, BPA interferes with biochemical mechanism useful to stabilize chromatin condensation of SPZ during the epididymal transit, by counteracting oxidation of thiol groups associated to chromatin.

5. Conclusions

The results of the current study support the hypothesis that endocrine-disrupting chemicals are important risk factors for declining male semen quality and suggest that environmental exposure to BPA may affect the main semen quality parameters. In the last few years, sperm quality parameters take on an important relevance in evaluation of gamete health. As an example, chromatin condensation and DNA damage in sperm cells [5, 40] are related each other, and DNA damage has been closely associated with poorer outcomes of numerous indicators of reproductive health, including lower fertilization, embryo quality, and implantation, and also with spontaneous abortion [41].

Of course, a clear understanding of BPA action mechanisms, including bioaccumulation, as well as of the presumed risks deriving from its exposure, is crucial to preserve male fertility. Moreover, given the complexity of BPA activity whose effects in animal models have been demonstrated at low and high doses [26], it is of critical public health importance to re-evaluate the current reference dose considered "safe" in humans [42].

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by Italian Ministry of University and Research (Grant PRIN to R. Pierantoni 2017), Università degli Studi della Campania (Grant Dip. Medicina Sperimentale-2017 to G. Cobellis), and Università degli Studi della Campania (Grant VALERE, Vanvitelli per la Ricerca 2019 to G. Cobellis).

References

[1] G. Cobellis, G. Cacciola, T. Chioccarelli et al., "Estrogen regulation of the male reproductive tract in the frog, *Rana esculenta*: a role in Fra-1 activation in peritubular myoid cells and in sperm release," *General and Comparative Endocrinology*, vol. 155, no. 3, pp. 838–846, 2008.

[2] R. A. Hess, "Spermatogenesis: an overview," in *Encyclopedia of Reproduction*, E. Knobil and J. D. Neill, Eds., pp. 539–545, Academic Press, Cambridge, MA, USA, 1999.

[3] G. Cobellis, G. Ricci, G. Cacciola et al., "A gradient of 2-arachidonoylglycerol regulates mouse epididymal sperm cell start-up," *Biology of Reproduction*, vol. 82, no. 2, pp. 451–458, 2010.

[4] R. Balhorn, M. Corzett, and J. A. Mazrimas, "Formation of intraprotamine disulfides in vitro," *Archives of Biochemistry and Biophysics*, vol. 296, no. 2, pp. 384–393, 1992.

[5] G. Cacciola, T. Chioccarelli, L. Altucci et al., "Low 17β-estradiol levels in CNR1 knock-out mice affect spermatid chromatin remodeling by interfering with chromatin reorganization," *Biology of Reproduction*, vol. 88, no. 6, p. 152, 2013.

[6] R. Chianese, T. Chioccarelli, G. Cacciola et al., "The contribution of lower vertebrate animal models in human reproduction research," *General and Comparative Endocrinology*, vol. 171, no. 1, pp. 17–27, 2011.

[7] C. Z. Yang, S. I. Yaniger, V. C. Jordan, D. J. Klein, and G. D. Bittner, "Most plastic products release estrogenic chemicals: a potential health problem that can be solved," *Environmental Health Perspectives*, vol. 119, no. 7, pp. 989–996, 2011.

[8] H. Hiroi, O. Tsutsumi, M. Momoeda, Y. Takai, Y. Osuga, and Y. Taketani, "Differential interactions of bisphenol A and 17β-estradiol with estrogen receptor alpha (ERα) and ERβ," *Endocrine Journal*, vol. 46, no. 6, pp. 773–778, 1999.

[9] L.-C. Xu, H. Sun, J.-F. Chen et al., "Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro," *Toxicology*, vol. 216, no. 2-3, pp. 197–203, 2005.

[10] D. Gentilcore, I. Porreca, F. Rizzo et al., "Bisphenol A interferes with thyroid specific gene expression," *Toxicology*, vol. 304, pp. 21–31, 2013.

[11] W. V. Welshons, S. C. Nagel, and F. S. vom Saal, "Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure," *Endocrinology*, vol. 147, no. 6, pp. S56–s69, 2006.

[12] A. Janesick and B. Blumberg, "Endocrine disrupting chemicals and the developmental programming of adipogenesis and obesity," *Birth Defects Research Part C: Embryo Today: Reviews*, vol. 93, no. 1, pp. 34–50, 2011.

[13] O. Takahashi and S. Oishi, "Disposition of orally administered 2,2-bis(4-hydroxyphenyl)propane (bisphenol A) in pregnant rats and the placental transfer to fetuses," *Environmental Health Perspectives*, vol. 108, no. 10, pp. 931–935, 2000.

[14] S. D. Yoo, B. S. Shin, B. M. Lee et al., "Bioavailability and mammary excretion of bisphenol a in Sprague-Dawley rats," *Journal of Toxicology and Environmental Health, Part A*, vol. 64, no. 5, pp. 417–426, 2001.

[15] Y. Ikezuki, O. Tsutsumi, Y. Takai, Y. Kamei, and Y. Taketani, "Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure," *Human Reproduction*, vol. 17, no. 11, pp. 2839–2841, 2002.

[16] G. Schönfelder, W. Wittfoht, H. Hopp, C. E. Talsness, M. Paul, and I. Chahoud, "Parent bisphenol A accumulation in the human maternal-fetal-placental unit," *Environmental Health Perspectives*, vol. 110, no. 11, pp. A703–A707, 2002.

[17] F. Cariati, N. D'Uonno, F. Borrillo, S. Iervolino, G. Galdiero, and R. Tomaiuolo, "Bisphenol a: an emerging threat to male fertility," *Reproductive Biology and Endocrinology*, vol. 17, no. 1, p. 6, 2019.

[18] M. S. Rahman, W.-S. Kwon, P. C. Karmakar, S.-J. Yoon, B.-Y. Ryu, and M.-G. Pang, "Gestational exposure to bisphenol A affects the function and proteome profile of F1 spermatozoa in adult mice," *Environmental Health Perspectives*, vol. 125, no. 2, pp. 238–245, 2017.

[19] A. Okada and O. Kai, "Effects of estradiol-17β and bisphenol A administered chronically to mice throughout pregnancy and lactation on the male pups' reproductive system," *Asian Journal of Andrology*, vol. 10, no. 2, pp. 271–276, 2008.

[20] S. Salian, T. Doshi, and G. Vanage, "Perinatal exposure of rats to bisphenol A affects the fertility of male offspring," *Life Sciences*, vol. 85, no. 21-22, pp. 742–752, 2009.

[21] J. Vitku, L. Sosvorova, T. Chlupacova et al., "Differences in bisphenol A and estrogen levels in the plasma and seminal plasma of men with different degrees of infertility," *Physiological Research*, vol. 64, no. Suppl 2, pp. S303–S311, 2015.

[22] M. M. Dobrzyńska and J. Radzikowska, "Genotoxicity and reproductive toxicity of bisphenol A and X-ray/bisphenol A combination in male mice," *Drug and Chemical Toxicology*, vol. 36, no. 1, pp. 19–26, 2013.

[23] X.-L. Liu, X.-Y. Chen, Z.-C. Wang, T. Shen, and H. Zhao, "Effects of exposure to bisphenol A during pregnancy and lactation on the testicular morphology and caspase-3 protein expression of ICR pups," *Biomedical Reports*, vol. 1, no. 3, pp. 420–424, 2013.

[24] H. J. Wu, C. Liu, W. X. Duan et al., "Melatonin ameliorates bisphenol A-induced DNA damage in the germ cells of adult male rats," *Mutation Research*, vol. 752, no. 1-2, pp. 57–67, 2013.

[25] M. S. Rahman, W. S. Kwon, J. S. Lee, S. J. Yoon, B. Y. Ryu, and M. G. Pang, "Bisphenol-A affects male fertility via fertility-related proteins in spermatozoa," *Scientific Reports*, vol. 5, no. 1, p. 9169, 2015.

[26] C. A. Richter, L. S. Birnbaum, F. Farabollini et al., "In vivo effects of bisphenol A in laboratory rodent studies," *Reproductive Toxicology*, vol. 24, no. 2, pp. 199–224, 2007.

[27] J. Miyawaki, K. Sakayama, H. Kato, H. Yamamoto, and H. Masuno, "Perinatal and postnatal exposure to bisphenol A increases adipose tissue mass and serum cholesterol level in mice," *Journal of Atherosclerosis and Thrombosis*, vol. 14, no. 5, pp. 245–252, 2007.

[28] A. Suglia, R. Chianese, M. Migliaccio et al., "Bisphenol A induces hypothalamic down-regulation of the the cannabinoid receptor 1 and anorexigenic effects in male mice," *Pharmacological Research*, vol. 113, pp. 376–383, 2016.

[29] T. Chioccarelli, G. Cacciola, L. Altucci et al., "Cannabinoid receptor 1 influences chromatin remodeling in mouse spermatids by affecting content of transition protein 2 mRNA and histone displacement," *Endocrinology*, vol. 151, no. 10, pp. 5017–5029, 2010.

[30] D. P. Evenson, L. K. Jost, D. Marshall et al., "Utility of sperm chromatin structure assay as a diagnostic tool in the human fertility clinic," *Human Reproduction*, vol. 14, no. 4, pp. 1039–1049, 1999.

[31] D. P. Evenson, K. L. Larson, and L. K. Jost, "Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques," *Journal of Andrology*, vol. 23, no. 1, pp. 25–43, 2002.

[32] S. Said, H. Funahashi, and K. Niwa, "DNA stability and thiol-disulphide status of rat sperm nuclei during epididymal maturation and penetration of oocytes," *Zygote*, vol. 7, no. 3, pp. 249–254, 1999.

[33] D. Tiwari and G. Vanage, "Mutagenic effect of Bisphenol A on adult rat male germ cells and their fertility," *Reproductive Toxicology*, vol. 40, pp. 60–68, 2013.

[34] L. Mita, A. Baldi, N. Diano et al., "Differential accumulation of BPA in some tissues of offspring of Balb-C mice exposed to different BPA doses," *Environmental Toxicology and Pharmacology*, vol. 33, no. 1, pp. 9–15, 2012.

[35] S. Errico, T. Chioccarelli, M. Moggio, N. Diano, and G. Cobellis, "A new LC-MS/MS method for simultaneous and quantitative detection of bisphenol-A and steroids in target tissues: a power tool to characterize the interference of bisphenol-A exposure on steroid levels," *Molecules*, vol. 25, no. 1, p. E48, 2019.

[36] M. E. Pinto, P. S. L. Vilamaior, S. R. Taboga, and R. M. Góes, "Exposure of young rats to high estrogen doses leads to degeneration of elongated spermatids," *Tissue and Cell*, vol. 40, no. 1, pp. 31–42, 2008.

[37] G. Shetty, H. Krishnamurthy, H. N. Krishnamurthy, A. S. Bhatnagar, and R. N. Moudgal, "Effect of estrogen deprivation on the reproductive physiology of male and

female primates," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 61, no. 3–6, pp. 157–166, 1997.

[38] G. Shetty, H. Krishnamurthy, H. N. Krishnamurthy, A. S. Bhatnagar, and N. R. Moudgal, "Effect of long-term treatment with aromatase inhibitor on testicular function of adult male bonnet monkeys (M. radiata)," *Steroids*, vol. 63, no. 7-8, pp. 414–420, 1998.

[39] R. Oliva, "Protamines and male infertility," *Human Reproduction Update*, vol. 12, no. 4, pp. 417–435, 2006.

[40] D. T. Carrell, B. R. Emery, and S. Hammoud, "Altered protamine expression and diminished spermatogenesis: what is the link?" *Human Reproduction Update*, vol. 13, no. 3, pp. 313–327, 2007.

[41] S. E. M. Lewis and R. J. Aitken, "DNA damage to spermatozoa has impacts on fertilization and pregnancy," *Cell and Tissue Research*, vol. 322, no. 1, pp. 33–41, 2005.

[42] F. S. vom Saal and C. Hughes, "An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment," *Environmental Health Perspectives*, vol. 113, no. 8, pp. 926–933, 2005.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 1814867, 6 pages
https://doi.org/10.1155/2020/1814867

Research Article

Gestational Exposure to Synthetic Steroid Hormones Impaired Sperm Quantity and Quality in Wistar Rats

Samy Ismail Ahmed,[1] **Aamir Magzoub**,[2] **Mohammed Saeed Zayed Al-Ayed**,[3] **Gamal Ali Attia**,[4] **Basel A. Abdel-Wahab**,[5] **Masood M. Khateeb**,[6] **Asim M. Abdalla**,[7] **Heitham M. Mohammed**,[7] **Aymen N. Eldeen A. Elkareem**,[1] **and Ali Gadkarim A. Salih**[7]

[1]*Department of Anatomy, College of Medicine, Najran University, Najran, Saudi Arabia*
[2]*Department of Physiology, College of Medicine, Najran University, Najran, Saudi Arabia*
[3]*Department of Paediatrics, College of Medicine, Najran University, Najran, Saudi Arabia*
[4]*Department of Anatomy, College of Medicine, Assiut University, Assiut, Egypt*
[5]*Department of Pharmacology, College of Medicine, Assiut University, Assiut, Egypt*
[6]*Department of Pharmacology, College of Medicine, Najran University, Najran, Saudi Arabia*
[7]*Department of Anatomy, College of Medicine, King Khalid University, Abha, Saudi Arabia*

Correspondence should be addressed to Samy Ismail Ahmed; samyanatomist@yahoo.com

Received 31 August 2019; Revised 6 November 2019; Accepted 12 December 2019; Published 25 January 2020

Guest Editor: Rosanna Chianese

This study was designed to investigate the effect of prenatal exposure to synthetic sex steroid on sperm quantity and quality, relative testicular and epididymal weights, and reproductive hormones level in adult Wistar rats. Forty male Wistar rats were divided into two groups: a test group ($n = 20$) that included mature rats that were born to dams exposed to gestational treatment with hydroxyprogesterone and a control group ($n = 20$) that included mature rats born to untreated dams. Compared to the control group, the test group showed a significant reduction in the sperm count, viability and motility, relative testicular and epididymal weights together with increased abnormal spermatozoa ($p < 0.001$). The reproductive hormonal assay revealed significantly lower serum testosterone and higher levels of FSH and LH among the test groups compared to the control ($p < 0.05$ for all). Prenatal exposure to synthetic progesterone negatively affected sperm production and function, relative testicular and epididymal weights, and reproductive hormone levels.

1. Introduction

Recently, and with a significant progressive increase in the incidence of human infertility and testicular disorders, considerable concern has been expressed over the possibility that prenatal exposure to excess synthetic sex hormones and steroids may adversely affect male reproduction in both animals and humans [1, 2].

Clinical and experimental reports support the hypothesis that the fetal origin of testicular disorders is associated with exposure to endocrine disrupters. New data reported that prenatal exposure to such hormones is likely to cause testicular developmental disorders and, consequently, induce infertility [3–5]. Nevertheless, prenatal exposure to progesterone was reported to induce irreversible testicular growth alteration, reduced sperm production, and suppressed steroidogenesis [5, 6].

Hydroxyprogesterone is synthetic progesterone prescribed traditionally for many obstetric and nonobstetric conditions. In obstetrics, the hormone is used for the treatment of abnormal uterine bleeding, threatened miscarriages during the first trimester, primary infertility cases, preterm delivery prevention, and to prevent and treat abnormal endometrial thickening (endometrial hyperplasia). Nonobstetric uses include treatment of mammary pain in women with noncancerous breast disease and as a topical treatment in certain skin diseases [7–11].

Experimental studies showed that exposure to such drugs during the embryonic period of development that is marked as a critical period might significantly decrease the epididymal sperm count and sperm motility in rats. These abnormalities may result from alteration of the normal endocrine system function of the animal and interferences with synthesis, secretion, transport, metabolism, binding, and elimination of natural blood hormones that are responsible for homeostasis, reproduction, and developmental process [11, 12].

Following exposure to hydroxyprogesterone, recent evidence revealed abnormal morphometric and histological changes, including a reduction in sperm count, damaged seminiferous tubules, and decreased testicular and epididymal weights [7, 8, 11].

Although many experimental protocols extensively studied the impacts of prenatal exposure to estrogenic steroids on male and female reproduction, data are scarce concerning the male reproductive changes following prenatal exposure to progestogens. In this context, the present study was conducted to further explore the effects of prenatal exposure to hydroxyprogesterone on sperm quantity, sperm quality, relative testicular and epididymal weights, and reproductive hormone level using an experimental approach.

2. Material and Methods

2.1. Chemicals. Synthetic progesterone, hydroxyprogesterone caproate (Hydroxyprogesterone; Schering AG; Germany. Trade name: Proluton Depo ®), is available in an oily solution diluted by pure Spanish olive oil (1 : 4 ml). It was obtained from Najran Maternity Hospital.

2.2. Animals. Eight to ten weeks' old, pregnant females were divided into two groups and kept separate away from any stress in sterilized polypropylene cages (90 cm × 45 cm × 15 cm) lined with woody husk at 12 : 12 h light/dark cycle, (28 ± 7) ˚C temperature, fed on a commercial pellet and offered water ad libitum. Group one ($n = 10$) served as a control, and group two as a test group ($n = 10$). The females in the test group were subcutaneously injected with 10 mg/kg of hydroxyprogesterone on the 1st, 7th, and 14th day of gestation. The females in the control group were injected with a similar dose of a placebo (pure Spanish olive oil).

Twenty male puppies born to each of the test and control groups were randomly selected and allowed to grow for 90 days where they reached maturity. The test group included male rats that were born to dams treated with synthetic progesterone during pregnancy, whereas those who born to untreated dams served as a control ($n = 20$ in each group). The dams' body weights were taken daily to adjust the dose [8, 11, 13]. The dose used in this study was in the range of regular clinical use and the administrative schedule is similar to humans during pregnancy [11].

2.3. Tissue Collection and Preparation. Rats were anesthetized with chloroform, sacrificed by cervical dislocation, and blood samples were collected from each animal through cardiac puncture using a 3 ml syringe and placed in 5 ml plain blood collection tube to determine the serum levels of testosterone, luteinizing, and follicle-stimulating hormones.

The right testis and epididymis of each rat were quickly removed and cleaned from surrounding connective tissues and then weighed. The mid-to-distal region of the epididymis was perforated in a Petri dish by 3 mL needle. The oozed sperms containing fluid was then diluted in 1 mL physiological saline solution (0.9% NaCl). The apparent sperm suspension was gently mixed and kept at 37˚C for 5 minutes to allow for the dispersion of sperms in the medium [4, 12]. After thorough mixing, the sperm suspension was used to assess the sperm count, motility, viability, and morphology. Later, relative testis and epididymis weights were calculated per final body weight [11, 14].

2.4. Semen Analysis

2.4.1. Sperm Counts. A drop of the diluted semen (1 : 20), thoroughly mixed, was transferred to a Neubauer hemocytometer using a micropipette, and a cover glass overlaid. The total number of the sperms was then observed and counted under a Carl Zeiss (Germany) Axio 2 Plus microscope at ×100 magnification. Sperms were counted in five small squares of the large central square, each square consisting of 16 smaller squares. Therefore, sperm concentration was expressed in terms of sperm X 10^6/ml. Two samples from each epididymis were examined, and the average counts were scheduled [12, 15–17].

2.4.2. Sperm Motility. The sperm motility was assayed microscopically within 5 minutes following their isolation from cauda epididymis at 37˚C. A drop of sperm suspension was placed on a Neubauer hemocytometer using a micropipette and then observed under a Carl Zeiss (Germany) Axio 2 Plus microscope at ×100 magnification. Sperm with any of the different motility types was recorded as motile sperm. Sperm motility was expressed as a percentage of motile sperm of the 200 sperm counted in 10 randomly selected fields for each rat [4, 11].

For each animal, two separate hanging drops were prepared to obtain the average.

2.4.3. Sperm Viability. This technique was used to differentiate between live and dead sperms. A drop of the diluted semen was transferred to an Eppendorf tube (1 mL) containing one drop of 1% Eosin stain. The contents were mixed gently, left for 5 minutes at 37˚C, and about 10 μL of the sample was then observed under a Carl Zeiss (Germany) Axio 2 Plus microscope at ×200 magnification. The head of dead spermatozoa was stained red while the live spermatozoa were unstained with Eosin. Sperm viability, expressed as a percentage of live sperms of the 200 sperms, was evaluated in 10 randomly selected fields for each rat [4, 11]. For each animal, two separate hanging drops were prepared,

and two independent observers assessed the viability. The data from each animal were used to obtain the average.

2.4.4. Sperm Morphology. A gently mixed drop of the sperm suspension in an Eppendorf tube (1 mL) was placed on a clean slide and gently spread to make a thin film. The film was air-dried [18] and then observed under a Carl Zeiss (Germany) Axio 2 Plus microscope using ×100 magnification. The relative percentages of abnormal sperms were counted from 10 different optical fields for each rat sample. Abnormal sperms included headless, tailless, and coiled tail [11, 13, 19]. For each animal, two separate hanging drops were prepared, and two independent observers assessed the abnormalities. The average results of each animal were obtained.

2.5. Serum Collection and Hormonal Assay. The blood serum was separated by centrifugation at 4,000 rpm for 5 minutes after overnight storage at 4°C and then stored at 20°C [8, 11, 12]. Specific commercially available ELISA kits, purchased from Elabscience Biotechnology Co., Ltd (Elabscience), China, were used to assess rat luteinizing hormone (LH) Catalog No: E-ELR0026, testosterone (Catalog No: E-EL-R0155) and follicle-stimulating hormone (FSH) (Catalog No: E-EL-R0391) serum level according to their manufacturer's method instructions listed on the following table (Table 1):

2.6. Statistical Analysis. The obtained data were analyzed using SPSS version16 (Chicago, USA) software program. Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to test the significant difference between different groups with the level of significance set at $p < 0.05$.

2.7. Ethical Approval. This study was committed to national and international ethical experimental protocols and was approved by college of medicine ethical approval committee—Najran University-KSA.

3. Results

3.1. Sperm Count. The mean epididymal sperm count per 0.1 g/epididymis of the test group was significantly lower from that of the control ($p < 0.001$) (Table 2), which reflects a clear difference in spermatozoa density between the progesterone-treated and the control groups.

3.2. Sperm Motility and Sperm Viability. The mean percentage of the sperm motility and viable spermatozoa showed a significant reduction in the test group compared to the control ($p < 0.001$) (Table 2, Figure 1).

3.3. Sperm Morphology. A significant increment in the total number of abnormal spermatozoa in the test group was observed compared to control. The mean percentages of the total and specific (headless, tailless, and coiled tail) abnormalities were significantly higher among the test group (Table 3, Figure 2).

3.4. Relative Testicular and Epididymal Weight. Compared with the control group, the mean percentage of relative testicular and epididymal weights of the test group showed a significant reduction ($p < 0.01$) (Table 4).

3.5. Hormonal Assay. Serum testosterone levels were significantly decreased ($p < 0.001$) in male rats born to dams treated with synthetic progesterone during pregnancy. However, serum FSH and LH levels were both significantly increased in the test group (Table 5).

4. Discussion

The present study showed interesting findings that uphold the previous studies [11, 12] in that prenatal exposure to synthetic progesterone negatively affected sperm production, sperm function, and testicular hormone levels.

The sperm count, motility, and viability were reduced significantly among the progesterone-treated (test) group compared to the control one, an effect that might be due to hormonal-induced abnormal alteration in the testicular structure and function during the embryonic period, which in turn affected sperm development and maturation. These findings were consistent with few previously reported findings that investigated the effects of prenatal exposure to progesterone on male mice and rat reproduction, respectively [11, 12]. Nevertheless, the present study boosts the fact that prenatal exposure to such a hormone induces long-term abnormalities on testicular histology, and confirmed a significant change in weights of the reproductive organs, sperm quantity and quality, serum reproductive hormone concentrations, and fertility at maturity following prenatal and neonatal rats' exposure to contraceptive compounds [20]. The same findings were reported after the gestational and lactational exposure of male mice to diethylstilbestrol [21].

On the other hand, the significant increment in the percentages of the total abnormal spermatozoa, headless, tailless, and coiled tail spermatozoa in the test group compared to the control suggested that administration of progesterone during pregnancy might also disrupt sperm quality and therefore may impair fertility [1, 20].

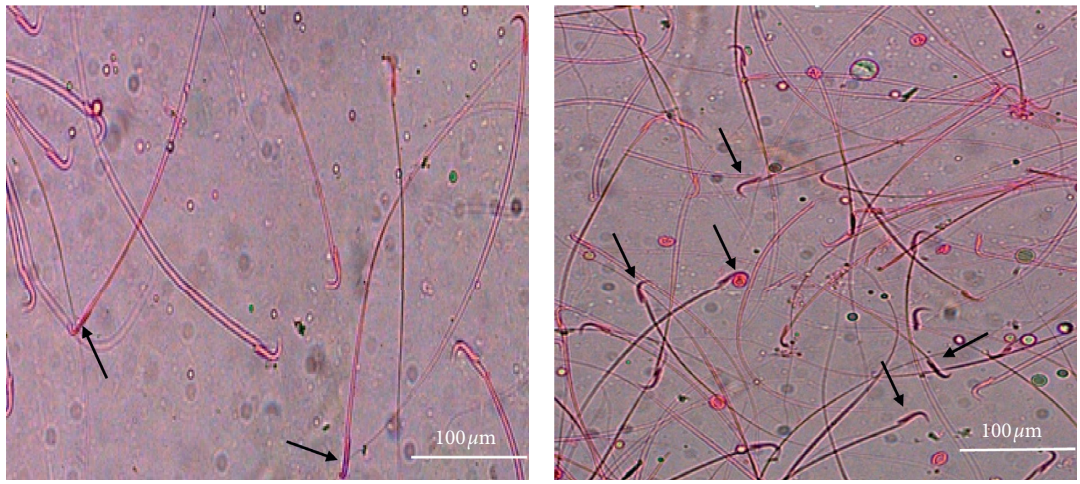
A significant decrease in the relative testicular and epididymal weights ($p < 0.01$) and in the test group compared to control was documented in the present study. This result might be due to germinal and somatic cell loss or hypotrophy, shrinkage of seminiferous tubules, decrease in the sperm count, and increase in the sperm abnormalities (headless and tailless) due to prenatal progesterone exposure. These findings are consistent with similar studies [5, 22]. The weight of the testis is mainly dependent on the mass of differentiated spermatogenic cells, and it has been used as a measure of spermatogenesis in rats [2]. A positive correlation between the weight of testis and the number of germ cells also was observed [8, 23].

Table 1

Hormone	Sensitivity	Intra-assay coefficients of variability Midrange (%)	Interassay coefficients of variability Midrange (%)
LH (mIU/mL)	0.94	4.55	5.96
FSH (ng/ml)	1.88	1.88	5.6
Testosterone (ng/ml)	0.17	6.72	6.18

Table 2: Mean percentage of the sperm count, motility, and viability in the control and test groups.

Parameter	Control	Test group	p value
Sperm count (million/ml)	(113.55 ± 10.46)	(81.72 ± 5.61)	$(p < 0.001)$
Motility (%)	(82.42 ± 6.62)	(63.65 ± 6.49)	$(p < 0.001)$
Viability (%)	(81.82 ± 6.54)	(62.90 ± 6.10)	$(p < 0.001)$



(a) (b)

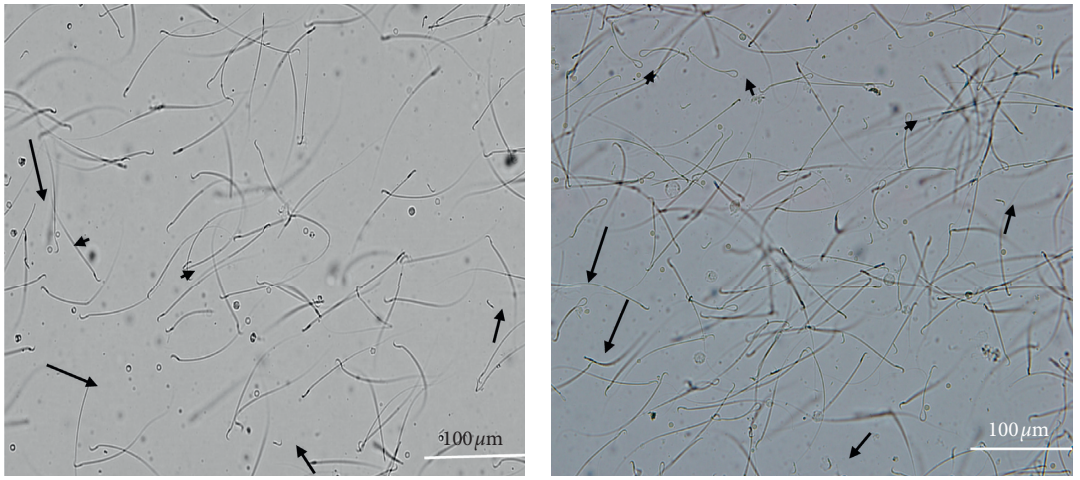
Figure 1: Micrographs illustrating sperm viability: control group (a), and the test group (b). Note the difference in the number of dark-stained dead sperms (yellow arrows) (×200).

Table 3: Mean percentage of total and specific abnormalities in the control and test groups.

Parameter	Control group	Test group	p value
Total sperm abnormality (%)	(15.10 ± 1.42)	(41.16 ± 3.93)	$(p < 0.005)$
Headless	$(1.42 \pm .41)$	(9.78 ± 1.05)	$(p < 0.005)$
Tailless	$(1.91 \pm .48)$	(16.42 ± 2.78)	$(p < 0.005)$
Coiled tail	$(2.86 \pm .52)$	(16.38 ± 3.89)	$(p < 0.001)$

The present study also included the effect of prenatal progesterone treatment on the reproductive hormones, namely testosterone, LH, and FSH serum levels. Compared to controls, a significant reduction in the serum testosterone level as well as a significant increase in the serum FSH and LH level were observed in the test group. Similar findings were reported in rats following prenatal exposure to hydroxyprogesterone [11, 12] as well as neonatal exposure to estrogen [24, 25]. The reduction in serum testosterone levels in the test group might be explained by a reduction in the Leydig cell count, diminished responsiveness of Leydig cells to LH, or direct inhibition of testicular steroidogenesis [5, 25]. A significant decrease in the 3b-hydroxysteroid dehydrogenase (3b-HSD) and 17b-hydroxysteroid dehydrogenase (17b-HSD) activities and steroidogenesis in the testes of mice prenatally exposed to hydroxyprogesterone was reported [6]. As testosterone is essential for the normal development of sperms in terms of different stages in the spermatogenesis process, the low hormone levels in the test group are expected to contribute to the observed reduction in the sperm count. The high serum FSH levels observed in the test group could be due to germ cell loss in the spermatogenic compartment or damage to the Sertoli cells, which is expected to decrease inhibin hormone, thereby affecting the negative feedback regulation of FSH secretion.

Similarly, the increased levels of LH, together with decreased levels of serum testosterone in the test groups, are indicative of loss of the negative feedback of testosterone on LH secretion caused by impairment of Leydig cell structure or function. Earlier studies reported that neonatal exposure to diethylstilbestrol resulted in suppression of androgen action in addition to abnormalities in the male reproductive tract. Nevertheless, the increased LH serum level delayed the onset of mesenchymal cell differentiation into Leydig cells [12, 22, 26, 27].



(a) (b)

Figure 2: Micrographs showing sperms with abnormal morphologies including headless (long arrow), tailless (short arrow), and coiled tail (arrowhead) in the control (a) and test groups (b) (×100). Note that the number of abnormal sperms is higher in the test group compared to the control.

Table 4: Effects of synthetic progesterone on the testicular and epididymal relative weights (g).

Parameter	Control	Test I	p value
Relative testicular weight	(0.011 ± 0.0002)	(0.0062 ± 0.0002)	$(p < 0.001)$
Relative epididymal weight	(0.004 ± 0.001)	(0.002 ± 0.0002)	$(p < 0.001)$

Table 5: Mean serum reproductive hormones (ng/ml) in the control and test groups.

Parameter	Control	Test group	p value
LH	(3.13 ± 0.10)	(3.9 ± 0.10)	$p < 0.01$
FSH	(3.90 ± 0.14)	(5.0 ± 0.10)	$p < 0.01$
Testosterone	(2.33 ± 0.09)	(1.04 ± 0.06)	$p < 0.01$

In conclusion, the present study showed that prenatal exposure to synthetic progesterone adversely affected sperm production and function, relative testicular and epididymal weights, and reproductive hormone levels. These findings raise a question on the safety profile of progesterone use during pregnancy, particularly in the presence of any testicular abnormality.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] E. Carlsen, A. Giwercman, N. Keiding, and N. E. Skakkebaek, "Evidence for decreasing quality of semen during past 50 years," *BMJ*, vol. 305, no. 6854, pp. 609–613, 1992.

[2] R. J. Kavlock, G. P. Daston, C. DeRosa et al., "Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop," *Environmental Health Perspectives*, vol. 104, no. Suppl4, pp. 715–740, 1996.

[3] D. G′eraldine, C. Levacher, and H. Rene, "Estrogen effects on fetal and neonatal testicular development," *Reproduction*, vol. 132, no. 4, pp. 527–538, 2006.

[4] T. Pushpalatha, P. R. Reddy, and P. S. Reddy, "Gestational exposure to hydroxyprogesterone caproate suppresses reproductive potential in male rats," *Naturwissenschaften*, vol. 92, no. 8, pp. 385–388, 2005.

[5] H. O. Goyal, A. Robateau, T. D. Braden, C. S. Williams, K. K. Srivastava, and K. Ali, "Neonatal estrogen exposure of male rats alters reproductive functions at Adulthood1," *Biology of Reproduction*, vol. 68, no. 6, pp. 2081–2091, 2003.

[6] T. Pushpalatha, P. R. Reddy, and P. S. Reddy, "Effect of prenatal exposure to hydroxyprogesterone on steroidogenic enzymes in male rats," *Naturwissenschaften*, vol. 90, no. 1, pp. 40–43, 2002.

[7] S. I. Ahmed, T. O. Ali, and A. S. Elsheikh, "Ultra-structure of testes of rats born to dams treated with hydroxy-progesterone hexanoate," *Asian Pacific Journal of Reproduction*, vol. 5, no. 6, pp. 510–513, 2016.

[8] S. I. Ahmed, A. S. Elsheikh, G. A. Attia, and T. O. Ali, "Prenatal progesterone exposure of male rats induces morphometric and histological changes in testes," *Asian Pacific Journal of Reproduction*, vol. 5, no. 3, pp. 204–209, 2016.

[9] P. Marianowski and E. Radwańska, "Intramuscular vs vaginal progesterone for luteal support in cycles of in vitro fertilization," *Ginekologia polska*, vol. 71, no. 9, pp. 1064–1070, 2000.

[10] A. Vidaeff and M. A. Belfort, "Critical appraisal of the efficacy, safety, and patient acceptability of hydroxyprogesterone caproate injection to reduce the risk of preterm birth," *Patient Preference and Adherence*, vol. 2013, pp. 683–691, 2013.

[11] C. Harini, S. B. Sainath, and P. S. Reddy, "Recovery of suppressed male reproduction in mice exposed to progesterone during embryonic development by testosterone," *Reproduction*, vol. 137, no. 3, pp. 439–448, 2009.

[12] T. Pushpalatha, P. Ramachandra Reddy, and P. Sreenivasula Reddy, "Impairment of male reproduction in adult rats exposed to hydroxyprogesterone caproate in utero," *Naturwissenschaften*, vol. 91, no. 5, pp. 242–244, 2004.

[13] T. Pushpalatha, P. R. Reddy, G. Trivikram, and P. S. Reddy, "Reduced spermatogenesis in rats exposed transplacentally to hydroxyprogesterone," *Cytologia*, vol. 68, no. 4, pp. 369–373, 2003.

[14] J. D. Bancroft and M. Gamble, *Theory and Practice of Histological Techniques*, Churchill Livingstone, London, UK, 5th edition, 2002.

[15] M. H. Feustan, K. R. Bodnai, and S. L. Kerstetter, "Reproductive toxicity of 2-methoxy ethanol applied dermally to occluded and non-occluded sides in male rats," *Toxicology and Applied Pharmacology*, vol. 100, no. 1, pp. 145–165, 1989.

[16] R. E. Linder, L. F. Strader, and W. K. McElroy, "Measurement of epididymal sperm motility as a test variable in the rat," *Bulletin of Environmental Contamination and Toxicology*, vol. 36, no. 1, pp. 317–324, 1986.

[17] L. R. Fransa, M. O. Suescun, and J. R. Miranda, "Testis structure and function in a monogenetic hyperadipose rat model at prepubertal and adult ages," *Endocrinology*, vol. 147, no. 3, pp. 1556–1563, 2005.

[18] A. E.-K. Afaf, F. S. Afrah, I. S. Adel, and R. A. Mohamed, "Animal model study of reproductive toxicity of the chronic exposure of dicofol," *Life Science Journal*, vol. 6, no. 3, pp. 1–18, 2009.

[19] W. Pizzi, J. Barnhart, and D. Fanslow, "Monosodium glutamate admlinistration to the newborn reduces reproductive ability in female and male mice," *Science*, vol. 196, no. 4288, pp. 452–454, 1977.

[20] K. A. Thayer, R. L. Ruhlen, K. L. Howdeshell et al., "Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17α-ethinyl oestradiol," *Human Reproduction*, vol. 16, no. 5, pp. 988–996, 2001.

[21] M. R. Fielden, R. G. Halgren, C. J. Fong et al., "Gestational and lactational exposure of male mice to diethylstilbestrol causes long-term effects on the testis, sperm fertilizing abilityin vitro, and testicular gene expression," *Endocrinology*, vol. 143, no. 8, pp. 3044–3059, 2002.

[22] L. O'Donnell, K. M. Robertson, M. E. Jones, and E. R. Simpson, "Estrogen and spermatogenesis," *Endocrine Reviews*, vol. 22, no. 3, pp. 289–318, 2001.

[23] O. K. Schlappack, J. I. Delic, J. R. Harwood, and J. A. Stanley, "Protection from radiation-induced damage to spermatogenesis in the androgen pretreated rat," *Radiotherapy and Oncology*, vol. 12, no. 3, pp. 219–224, 1988.

[24] N. Atanassova, C. McKinnell, M. Walker et al., "Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in Adulthood1," *Endocrinology*, vol. 140, no. 11, pp. 5364–5373, 1999.

[25] C. McKinnell, N. Atanassova, K. Williams et al., "Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol," *Journal of Andrology*, vol. 22, no. 2, pp. 323–338, 2001.

[26] R. M. Sharpe, N. Atanassova, C. McKinnell et al., "Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: a possible role for estrogens in Sertoli cell Development1," *Biology of Reproduction*, vol. 59, no. 5, pp. 1084–1094, 1998.

[27] H. B. Siril Ariyaratne, J. Ian Mason, and S. M. L. C. Mendis-Handagama, "Effects of thyroid and luteinizing hormones on the onset of precursor cell differentiation into Leydig progenitor cells in the prepubertal rat Testis1," *Biology of Reproduction*, vol. 63, no. 3, pp. 898–904, 2000.