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Obstetrics and Gynecology: Open Access

Habermehl TL, et al. Gynecol Obstet Open Acc 02: OBOA-131.

Research Article

DOI: 10.29011/ 2577-2236.100031

Germ Cell Depletion Influenced Neuromuscular, Sensory, Renal and Metabolic Function in PostReproductive Female Mice

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Citation: Habermehl TL, Parkinson KC, Mason JB (2018) Germ Cell Depletion Influenced Neuromuscular, Sensory, Renal and Metabolic Function in PostReproductive Female Mice. Gynecol Obstet Open Acc 02: 131. DOI: 10.29011/ 2577-2236.100031

Received Date: 19 September, 2018; **Accepted Date:** 11 October, 2018; **Published Date:** 19 October, 2018

Abstract

Numerous observations suggest a relationship between reproduction and health. This relationship has been difficult to define, particularly in mammals. At menopause, germ cell-diminished ovaries are relatively senescent. In mice, replacement of these senescent ovaries with young, actively cycling ovaries significantly increased life and health span in recipients. While we were successful in extending life and health span in postreproductive females, these experiments were not designed to distinguish between the contributions of ovarian hormones from actively cycling germ cells and the somatic tissue components of the ovaries. In the current study, we used 4-vinylcyclohexene diepoxide to deplete ovarian germ cells in 28-day old CBA/J mice. These mice were evaluated at 13 months of age to determine if depletion of ovarian germ cells influenced health span. Germ cell-depletion significantly reduced musculoskeletal and renal function, but improved body composition at 13 months of age. Percent fat mass was reduced 9% and lean mass was increased 5% in 13-month old, germ cell-depleted mice. There was no significant age-associated change in glucose tolerance. However, germ cell-depleted mice displayed changes suggestive of early insulin resistance in response to a glucose tolerance test. Germ cell-depletion displayed a strong, positive trend on sensory function, but did not influence cognitive behavior or immune function. These finding suggest that the ovarian influence on health is not exclusive to the germ cells. Together, these findings provide strong incentive for further investigation of the influence of the reproductive and non-reproductive function of ovarian germ and somatic cells and hormones in the preservation of health.

Keywords: Body Composition; Cognitive Behavior; Health Span; Metabolism; Olfactory Ovarian; Senescence

Abbreviations

GD	:	Germ Cell Depleted
VCD	:	4-VinylCyclohexene Diepoxide
IP	:	Intraperitoneal
ICGT	:	Inverted- Cling Grip Test
PO	:	Oral
GTT	:	Glucose Tolerance Test
AUC	:	Area Under the Curve
MRI	:	Magnetic Resonance Imaging
BUN	:	Blood Urea Nitrogen

Introduction

Evidence over the past decade indicate that an individual's reproductive status is associated with an increased risk of developing chronic health conditions (NIH-NICHD, Research Priorities Bulletin, 2016). One study documented an association between shorter life spans and reproductive failure for a cohort of men [1]. The association is even more striking in women. Cardiovascular disease is rare in premenopausal women [2] but ovarian failure increases cardiovascular disease sharply at menopause [3] and in young women with premature ovarian failure [4]. Insulin resistance [5] and bone loss increase at menopause [6] and almost two-thirds of Americans with Alzheimer's disease are women [7]. The lack of mechanistic knowledge of how young ovaries protect young women from disease is a critical barrier to progress in this field. A

classic view of the initiating cause of menopause is the exhaustion of ovarian germ cells. However, removal of the germ cells, but retention of the somatic gonad in young invertebrates increases health and longevity [8,9]. In mammals, Dietary Restriction (DR) inactivates germ cells and extends health span [10,11]. Both these observations bring into question the commonly held views that 1) the function of gonadal somatic cells is solely to support germ cell maturation and that 2) ovarian hormones produced by actively cycling ovarian germ cells are essential for the maintenance of the female health advantage.

Previous work in our laboratory demonstrated that replacement of senescent ovaries in postreproductive female mice with young, actively-cycling ovaries resulted in an increase in life span and a decrease in cardiomyopathy and osteoarthritis [12-14]. However, the question remained; what was the role of ovarian germ cells and germ cell hormones in this ovarian-dependent extension of health? In the current study, to determine ovarian germ cell influence on health span in female mice, we chemically depleted ovarian germ cells in the ovaries of pre-pubertal mice and evaluated these mice along with mice that were not germ cell depleted with the mice reached a postreproductive age.

Material and Methods

Mice

Twenty-one-day old CBA/J strain female mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were housed in a standard laboratory animal environment (fresh filtered air, 15 changes/h; temperature, 21 ± 2˚C; humidity, 50 ± 20%; and light-dark cycle, 12:12 h). The mice were housed individually in ventilated cages (Green Line IVC Sealsafe Plus, Tecniplast, West Chester, PA, USA) on corncob bedding (7097 Corncob, Harlan Teklad, Bartonville, IL, USA) changed once a week, with added enrichment, in a specific-pathogen-free colony where pathology on sentinel mice was done quarterly and pathological results showed no breach in this status. The mice received deionized water and a certified laboratory diet ad libitum (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Bartonville, IL, USA). Mice were maintained in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved facility in accordance with the National Institutes of Health animal-use guidelines. Animal care and use protocols were developed under National Research Council guidelines found in the Guide for the Care and Use of Laboratory Animals. This project was approved by the Utah State University Institutional Animal Care and Use Committee.

Mice were euthanized by cervical dislocation. Immediately after cervical dislocation, athoracotomy was performed followed by rapid exsanguination via cardiocentesis. The heart and arterial tree were then removed from the mouse. Animals with acute, but not severe weight loss were treated with subcutaneous fluids and moistened food. Animals with acute, but not severe urine staining or rectal/vaginal prolapse were manually cleaned and treated with Desitin®. Mice were monitored at least twice daily, and weights were recorded monthly, more frequently when concerns arose. Aged, moribund mice found with overt clinical signs (catatonia) were euthanized. Criteria for euthanasia specific for aged mice were determined in coordination with the attending veterinarian and included, but were not limited to mice found in poor condition with or without crusting around the perineum and diarrhea, urine staining, persistent vaginal prolapse, chronic vulva/rectal swelling, kyphosis, respiratory distress, anorexia, poor coat condition and lack of grooming, moribund mentation, hind-limb weakness/ paresis, wounds not healing, limited mobility, neoplastic growth and unusual weight loss (or gain). Average weight loss in aged, female CBA/J mice, from peak weight to death is approximately 12% per month [15]. An increased rate of weight loss, but not total weight loss was the most critical factor for determining a moribund state. Unexpected deaths were uncommon, but included neoplastic growths (most commonly mammary), decubitus ulcers (extremely old animals) and uncontrolled cataleptic seizures (normally between 11-13 months of age).

Experimental Design

Animals were randomly assigned to control or germ cell depletion groups as follows (Figure 1).

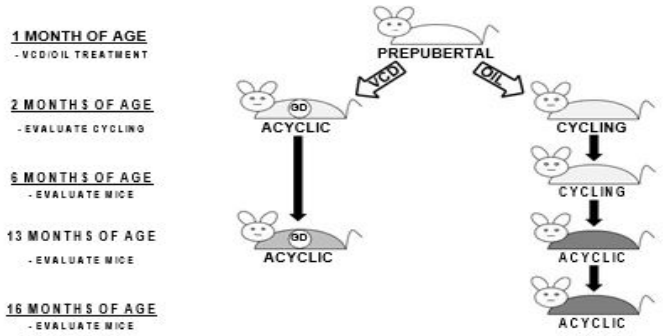


Figure 1: Mice began treatment with VCD at 28 days of age (for 15 days), were evaluated for reproductive cycling activity between 50-60 days of age and were collected at 13 months of age after the normal age of reproductive senescence in the control mice. Six-month old control mice (n=10), 13-month old oil-only treated control mice (n=45), 13-month old VCD treated mice (GD, n=5), 16-month old control mice (n=5).

Control Groups

Six-month old control mice: Reproductively cycling control mice were evaluated at six months of age**.**

Thirteen-month old control mice: Acyclic control mice were evaluated at 13 months of age. Thirteen-month old female mice are in a physiological transition state, which lasts approximately 100 days. Mice are transitioning from full exposure to cyclic ovarian hormones to cessation of hormone exposure at approximately 11 months of age

Sixteen-month old control mice: Acyclic control mice were evaluated at 16 months of age.

Germ Cell Depletion Group

Thirteen-month old VCD-treated mice: Intact animals were treated with 4-Vinylcyclohexene Diepoxide (VCD) at 28 days of age. Acyclic VCD-treated animals were evaluated at 13 months of age.

Age at Manipulation

Rodents do not undergo menopause, but instead have an estropause-like decrease in reproductive function. Female mice of the CBA /J strain become reproductively competent between 45 and 60 days of age. Initiation of germ cell depletion at 28 days of age was chosen to avoid major up-regulation of the reproductive system at the onset of puberty and to eliminate other influences the female gonad might have in addition to direct effects of gonadal hormones. These influences may include positive or negative feedback mechanisms, or system-wide ‘‘imprinting’’ influences the intact ovary may normally provide after 60 days of age. Reproductive decline in CBA/J mice usually begins with irregular cycles at 8-10 months of age. At 11 months of age, many CBA/J mice have become reproductively incompetent [16] with a complete loss of oocytes by 12 months of age. All 13-month old and all germ cell-depleted mice used in these experiments displayed a complete lack of reproductive cycling, as determined by vaginal cytology

Germ Cell Depletion

Intact animals at 28 days of age received daily Intraperitoneal (IP) injections of 160mg/kg VCD in sesame oil or sesame oil alone for 15 days. At 43 days of age, VCD and vehicle treatments were stopped. In our lab, treatment of 28-day old female CBA/J mice for 15 days with VCD results in cessation of reproductive cyclicity (persistent vaginal cornification), reduced ovarian weights (1.7mg in oil-only vs. 0.9mg in VCD-treated, P=0.030) and depleted primordial (P=0.004) and primary (P=0.029) ovarian follicles, compared with controls (Figure 2). VCD/oil treated animals were assayed at 13 months of age.

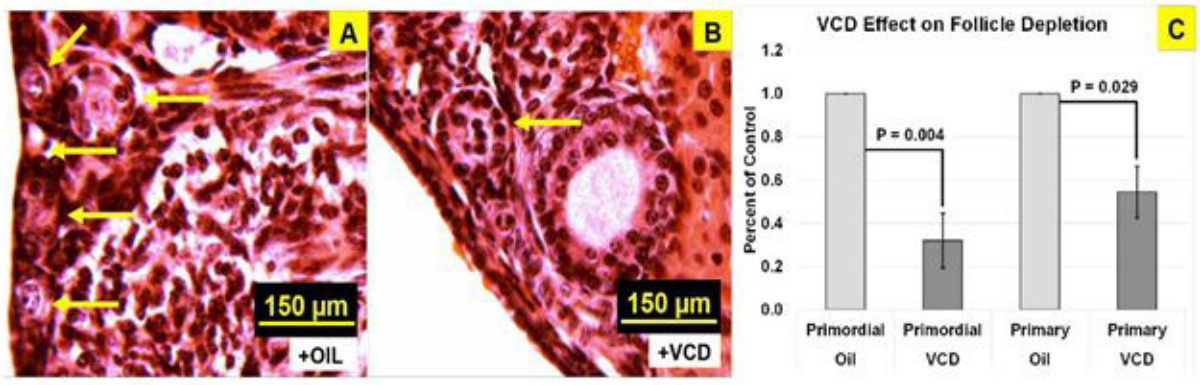


Figure 2: VCD depletion of small follicles in CBA/J mice. H&E-stained sections showing a) abundant small follicles in oil-treated (n=6) mice, b) reduced numbers of small follicles in VCD-treated (n=4) mice and c) and significant differences in both primordial and primary follicle numbers. Arrows indicate primordial and primary follicles.

Exclusion Criteria

Control mice that displayed cytological evidence of gonadal input at 13 months of age and VCD- treated mice that displayed cytological evidence of gonadal input at 60 days of age were excluded from analysis. Gonadal input was defined as cyclic changes on vaginal cytology, presumably due to cyclic influence of ovarian hormones. No gonadal input was defined as the lack of cyclic changes on vaginal cytology.

In Vivo Indices of Health Span

In vivo assays include measures of neuromuscular, sensory, cognitive, immune, renal and metabolic function and body composition.

Neuromuscular Function

The Inverted-Cling Grip Test (ICGT) is a measure of overall strength and muscular endurance of the mouse [17]. This test

consisted of placing the mouse on a cage-like wire grid and then inverting the grid over a padded surface. The testing device is a custom designed 43cm square of wire mesh consisting of 12mm squares of 1mm diameter wire, surrounded by a 4cm deep wooden border to prevent mice from climbing to the other side of the screen. Each mouse was placed in the center of the inverted screen, parallel to the floor. Next, the screen was tilted up 90˚ to cause the mouse to grab the screen tightly. The screen was subsequently turned another 90˚, again parallel with the floor, but flipped so that the mouse was positioned upside-down hanging suspended from the wire grid. The latency before the mouse lost its grip from the wire grid and fell to the padded surface at the bottom of the device was recorded to a maximum of 2h.

Sensory Function

The CBA/J strain of mice is homozygous for the retinal degeneration allele Pde6brd1, which causes blindness by approximately 21 days of age [18,19]. Because CBA/J mice become blind early in life, they rely more on olfactory, tactile, and auditory senses. We chose to use olfactory testing as a more sensitive measure of sensory function, compared with other tests, which may contain vision-dependent variables. Two separate tests of olfactory function were utilized in the current study: 1) a buried pellet test to assess olfactory identification and 2) a novel recognition block test to assess olfactory discrimination.

We started the olfactory identification test by familiarizing the mice with a sweetened cereal pellet by giving one pellet to each mouse for three days before the tests begin. Prior to test days, mice were fasted overnight for 12h. On test days, mice were moved to an isolated room where they could habituate for one hour. A clean cage was evenly filled with corncob bedding to 2cm deep for each trial and a sweetened pellet was buried at 0.5cm deep in a different location for each trial. Each mouse was placed in the middle of the cage and the time elapsed prior to pellet discovery was recorded (Figure 3). After testing, mice were returned to their home cage with ad libitum access to feed. Mice were tested for five consecutive days and maintained greater than 90% of their body weight throughout the five-day testing period. The olfactory discrimination test utilized wooden blocks with familiar (self) and novel (non-self) scents to test changes in olfactory perception. Each mouse was placed in a clean cage that contained three, individually labeled wooden blocks and after a one-hour habitation time, the three blocks were removed from each cage and placed in individually labeled bags along with a handful of bedding from each cage. These were used as 'Familiar' blocks for that mouse. Subsequently, mice were recorded for six, 30-second trials in a clean cage containing the familiar blocks and bedding collected during the habituation period. For the seventh trial, one of the original blocks was exchanged for a novel block from the cage of a different, unfamiliar mouse, leaving two familiar blocks and one novel block in the cage for an additional 30-second video recording. Recorded video was analyzed for 1) total time spent with or away from any of the blocks and 2) time spent with novel verses familiar blocks [20].

Figure 3: Buried pellet tests Mice could eat the food pellet after discovery.

Cognitive Behavior

Burrowing behavior was reported in a recent study, to suffer an age-related decline in mice at around nine months of age, with a further decline seen at 14 months [21]. To discriminate burrowing behavior from several similar behaviors, bedding was used instead of feed, or other foreign substances to isolate natural burrowing behavior without other motivators such as hunger or curiosity of a new substance. Burrowing behavior was tested by placing a plastic tube (5cm x 20cm) filled with bedding material into the home cage and quantifying the amount of bedding material removed at 15, 30 and 60 min and overnight. The mice were habituated to the testing apparatus by placing the empty tube into the home cage overnight. On the next day, mice were moved, along with their home cage to a dedicated behavioral testing room. The tubes were filled with 125gm of bedding material (corncob bedding) and the total weight of the tube and bedding were recorded. All mice were habituated for one hour in the testing room, after which all food, water and other enrichment items were removed, the apparatus was placed in the cage and a timer started. The weight of the tube and the bedding material that remained in the tube was checked at 15, 30 and 60min and overnight. Most of the material was removed and significant differences were most often found after the 15min time point, diminishing any potential disruptive influence of weighing at subsequent time points.

Metabolic Function

An Intraperitoneal Glucose Tolerance Test (IPGTT) was performed in mice that were feed- deprived for 4-5h. We chose an

IPGTT rather than an Oral (PO) GTT because the PO test increases stress on the tested subjects and is associated with gastrointestinal variability in the timing and dose of glucose absorbed. Variability in the IPGTT is often attributed to the injection needle penetrating the intestines and glucose being variably deposited into the gastrointestinal tract rather than the intraperitoneal cavity. We utilized short-needled (6mm) insulin syringes to minimize this possibility. We chose a 4-5h fast due to the metabolic differences between humans and mice and additionally because aged females do not respond well to an overnight fast [22]. Blood glucose levels were measured using Free Style Freedom Lite Blood Glucose Monitoring System (Abbott Diabetes Care Inc. Alameda, CA, USA). Blood droplets were obtained from a small nick at the tip of the tail at 2h prior to testing, immediately prior to glucose administration (t0), and at 15, 30, 60, and 120min after injection of 15% D- glucose (2.8g/kg lean body mass). Calibration of the Free Style Freedom Lite Blood Glucose Monitoring System was performed using control test solutions provided by the manufacturer. Glucose measurements were plotted as the mean glucose level for each cohort at each time point. The results were also expressed as the Area Under the Curve (AUC) for the 120min assay. The AUC corresponding to each animal was calculated by the trapezoid method [23], using as reference each individual baseline blood glucose measurement prior to glucose administration (t0). The sum of the trapezoidal areas between the 0, 15, 30, 60 and 120min time points corresponding to each animal was computed to obtain the AUC.

Immune Function

Changes in immune function were evaluated by measuring alterations in T-cell subsets present in the blood of mice from each group [24]. For the T-cell subset evaluation, approximately 200ul of whole blood was collected in heparin-treated blood tubes from the superficial temporal vein in mice after a 4-5h fast. Blood cells were processed and immunostained using a Mouse Naive/ Memory T Cell Panel, including anti-mouse CD4, anti-mouse CD3, anti-mouse CD62L and anti-mouse CD44 and stained with a Fixable Live/Dead viability dye. Flow cytometric analysis was performed using standard settings on a SORP FACSAria II Flow Cytometer. Samples were analyzed using FACSDiva Version 6.1.3. and reported as CD4+ and CD8+ central naive, central memory, peripheral naive and peripheral memory T cells and the ratio of naive to memory cells (Figure 4).

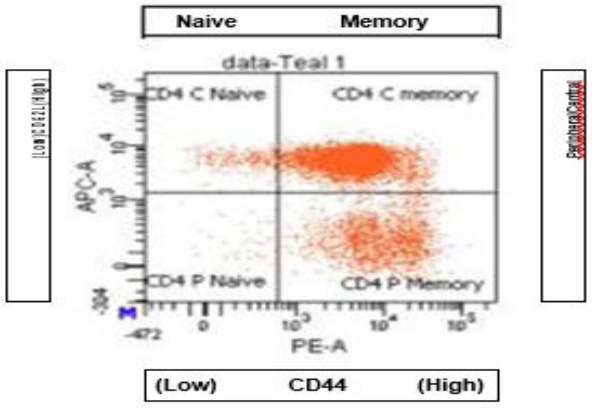


Figure 4: T-cell subset assay: central naive cells were selected with high CD62 L and low CD44, central memory cells with high CD62 L and high CD44, peripheral naive cells were selected with low CD62 L and low CD44 and peripheral memory cells with low CD62 L and high CD44.

Renal Function

Kidney function was evaluated using a serum renal panel at the Utah Veterinary Diagnostic Laboratory. An automated wet biochemistry analyzer (Dimension Xpand Plus, Siemens Healthcare Diagnostics Inc., Newark, DE, USA) was used to analyze serum samples for levels of Blood Urea Nitrogen (BUN), creatinine, sodium, potassium, chloride, magnesium, glucose, phosphorus, calcium, total protein and albumin. Blood urea nitrogen was analyzed using the Urease method, creatinine using a modified kinetic Jaffe method, magnesium using Methylthymol Blue (MTB) and phosphorus using a modified phosphomolybdate method. Precision control level 2 (human serum) and chemistry control level 3 (Randox Laboratories Limited, Crumlin County, Antrim, UK, Control level 2 #UN1557, Control level 3 #UE1558) were run on each day of serum sample testing. These two levels of control were reconstituted and stored according to manufacturer instructions.

Body Composition

Normal aging in humans and rodents is characterized by a reduction in skeletal muscle mass and total body water, as well as increases in adipose tissue mass. Alterations in mouse whole body composition was assessed using an EchoMRI-700

Magnetic Resonance Imaging system (EchoMRI, Houston, TX, USA). The EchoMRI-700 system is housed in a dedicated area to minimize stress and the entry of disease or contaminants. Prior to each run, the system was calibrated using a standard provided by Echo Medical System. Each mouse was weighed and placed into an appropriate size tube. The tube was then being placed in the EchoMRI-700 machine for measurements (1min). The output information included lean tissue mass, fat mass, free water and total body water. No anesthesia was required for this procedure [25].

Ex Vivo Anatomic Indices of Health

Kidney, spleen, heart and body weights were collected at necropsy. Organ weights were normalized to body weight

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 7.01 (Graph Pad Software, Inc. La Jolla, CA). A D'Agostino-Pearson omnibus test was used to determine normality. Data were analyzed with two-factor ANOVA and a Tukey-Kramer post-hoc test was used to determine difference between groups. Individual treatments were further analyzed by paired Student's t- test, two-tailed, unequal distribution of variance assumed. Test results were considered significant for P Values $P<0.05$.

Results

Neuromuscular Function

Performance on the ICGT, defined as the average time (duration in seconds) before falling from the grid, declined with age and with germ cell depletion. The effect of age on overall muscle strength, as measured by the ICGT, is summarized in (Figure 5). A significant age-related decline in grip strength was found in 16-month old control mice (45% reduction) and tended to be reduced in 13-month old mice (25% reduction). Thirteen-month old germ cell-depleted mice fell significantly sooner (35%) than the 13-month old control mice.

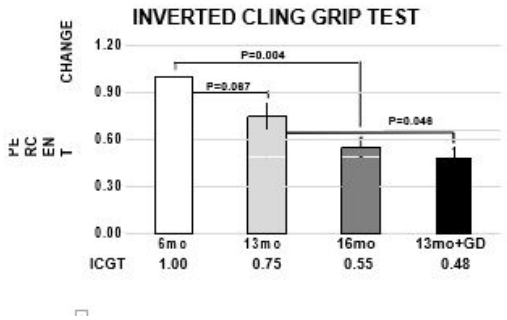


Figure 5: Inverted Cling Grip Test. Grip strength decreased significantly with age. Grip strength was also significantly decreased by germ cell depletion (13mo+GD). Values are reported as a percentage of the values in adult, six-month old mice.

Sensory Function

Time to pellet discovery in the Buried Pellet Test (olfactory identification) was strongly influenced by age, and mildly by germ cell depletion in female CBA/J mice (Figure 6). Sixteen- month old control mice tended (P=0.052) to take longer to pellet discovery than six-month old control mice. Germ cell-depleted 13-month old mice found the buried pellet 12% faster than 13- month old control mice.

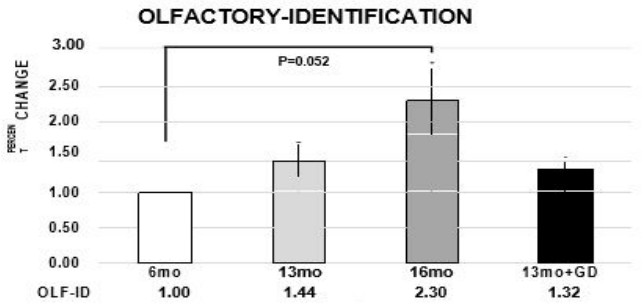


Figure 6: Olfactory Identification (OLF-ID). In the buried pellet test, age strongly influenced time to pellet discovery. Germ cell depletion did not significantly influence this trend in 13-month old mice. Values are reported as a percentage of the values in adult, six-month old control mice.

Novel Recognition Block Test (Olfactory Discrimination).

Both time spent exploring the novel block and time spent exploring familiar blocks increased with age (Figure 7). Time spent exploring the novel blocks and time spent exploring familiar blocks were strongly correlated. Germ cell depletion in 13-month old mice led to increased time spent exploring novel blocks (41%) and decreased time on all blocks (10%), compared with 13- month old control mice (Figure 7). Olfactory discrimination correlations. In the block test, time spent exploring novel blocks was significantly correlated with time spent exploring familiar blocks (Spearman correlation, $r=0.776$, $p= 0.004$) and time spent exploring all blocks (Spearman correlation, $r=0.951$, $p<0.001$). Time on all blocks increased with age but was decreased 10% by germ cell depletion.

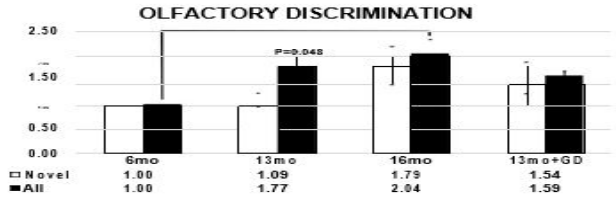


Figure 7: Olfactory discrimination correlations. In the block test, time

spent exploring novel blocks was significantly correlated with time spent exploring familiar blocks (Spearman correlation, r=0.776, p= 0.004) and time spent exploring all blocks (Spearman correlation, r=0.951, p<0.001). Time on all blocks increased with age but was decreased 10% by germ cell depletion.

Cognitive behavior

In the Burrowing test, 16-month old mice removed significantly less bedding in 15 minutes than 6-month old control mice and tended to remove less than 13-month old mice (P=0.053). Germ cell-depleted 13-month old mice were not different from 13-month old control mice (Figure 8).

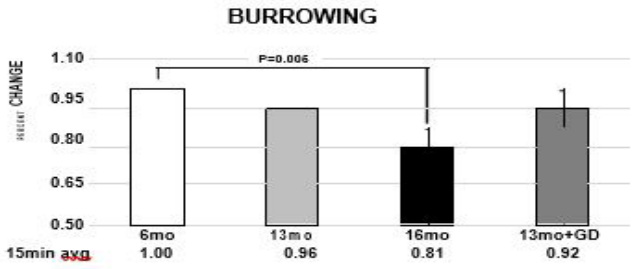
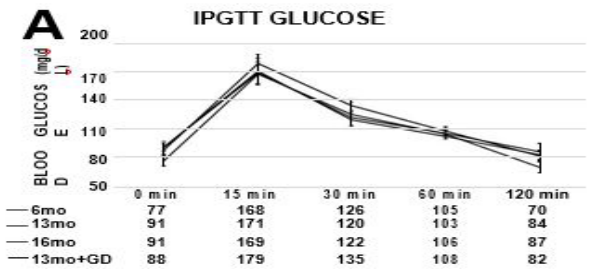


Figure 8: Cognitive function. In the burrowing assay, age significantly decreased the amount of bedding removed in 15 minutes (15min avg). The amount of bedding removed by 13-month old mice was slightly (4%) decreased by germ cell-depletion. Values are reported as a percentage of the values in adult, six-month old mice (6mo = 0.93% of bedding removed in 15 minutes).

Metabolic Function

Glucose measurements at each time point were plotted as the mean glucose level for each cohort (Figure 9A). Because all groups did not have equivalent fasting glucose levels, results were also expressed as the AUC for the 120-minute assay (Figure 9B). Area under the curve calculations show that during IPGTT, in 13-month old mice, germ cell depletion tended to increase the AUC (5%) and increased the blood glucose level at 30min (12%), compared with 3-month old control mice. There was no significant age-associated change in glucose tolerance, compared with six-month old mice.



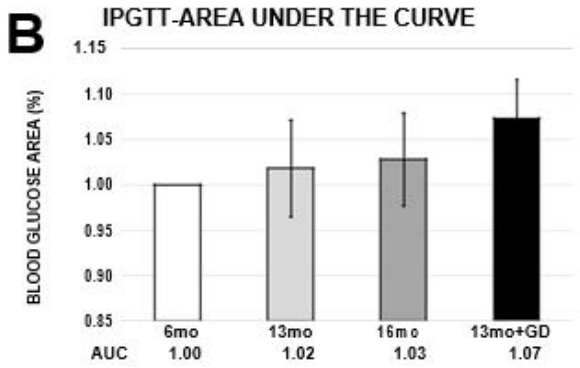


Figure 9(A-B): Metabolic function. The IPGTT assay detected no significant difference in glucose tolerance between six, 13- and 16-month old mice. Germ cell depletion had a modest influence on this parameter. Values are reported as A) blood glucose values for group glucose curves and for B) the area under the curve, as a percentage of the values in six-month old mice.

Immune Function

The ratio of central naive cells (high CD62 L and low CD44) to central memory cells (high CD62 L and high CD44), which normally decreases with aging, was used as a measure of immune function (Figure 10). In 13- and 16-month-old females, the ratio of naive T cells to memory T cells was negatively influenced by aging. Germ cell depletion had no significant influence on T-cell subsets in 13-month old mice.

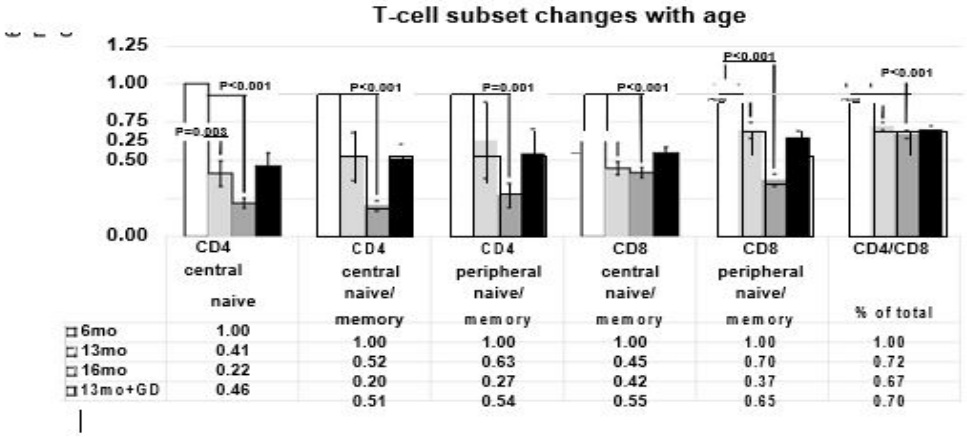


Figure 10: T-cell subset changes with age: Both CD4+ and CD8+ naive cells decreased with age. This is demonstrated by decreased central naive cells and decreased naive to memory cell ratio in both CD4+ and CD8+ populations. The percentage of CD4+ T cells as a percentage of total T cells decreased as well. Germ cell depletion had no significant influence on T-cell subsets in 13-month old mice. Data is reported as a percentage of values in adult, six-month old mice.

Kidney Function

Values for urine specific gravity and serum sodium, potassium and chloride (mEq/L) and total protein and albumin (g/dL) in 13- and 16-month old control mice were not significantly changed from values in six-month old control mice. Glucose, magnesium, phosphorus and calcium (mg/dL) were modestly decreased in 13- and 16-month old control mice from the levels seen in six-month old control mice. Blood Urea Nitrogen (BUN) was slightly increased at 13 months of age and slightly decreased at 16 months of age (Figure 11). Creatinine was reduced in 13- and 16-month old controls, compared with six-month old mice (16% and 21%, respectively). Germ cell depletion significantly reduced serum creatinine in 13-month old mice. The BUN: Cre ratio (Blood Urea Nitrogen: Creatinine) was increased in the 13-month old control group (33%). By 16 months of age, the BUN: Cre ratio increase was only 16% in the 16-month old control group. Germ cell depletion tended to increase the BUN: Cre ratio at 13 months of age.

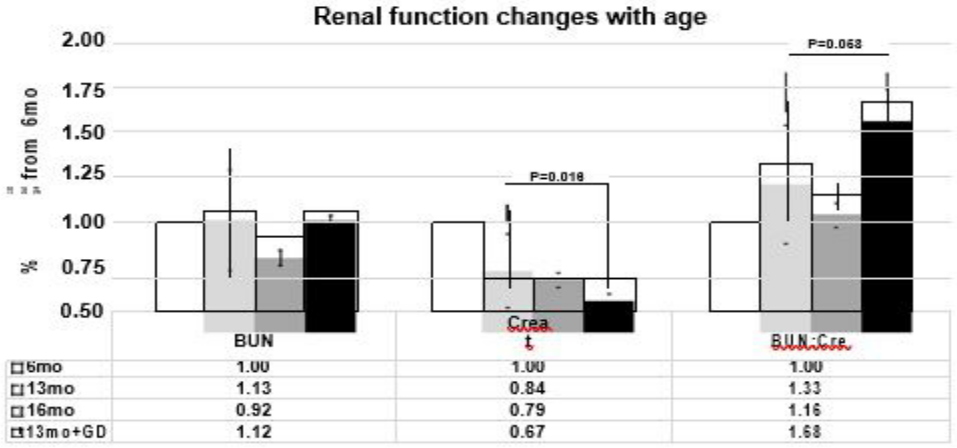


Figure 11: Renal function. Serum creatinine levels were significantly decreased with age and further decreased by germ cell depletion in 13-month old mice. The BUN: Cre ratio as a measure of renal function tended to be increased by germ cell depletion. Data is reported as a percentage of values in adult, 6-month-old mice.

Body Composition-MRI

There were significant differences in body weight between age groups, but no difference due to treatment (Figure 12). Fat mass was significantly increased at 13 and 16 months of age in control mice. However, half of the gain in fat mass at 13 months of age was lost by 16 months of age (P=0.006). Fat mass was reduced 9% by germ cell depletion in 13-month old mice. The percentage of lean body mass and total body water were significantly reduced in 13-month old control mice. Most of these loses were regained by 16 months of age. Lean mass and total body 448water were modestly increased by 449germ cell depletion in 13-month old 450mice (5% and 6%, respectively).

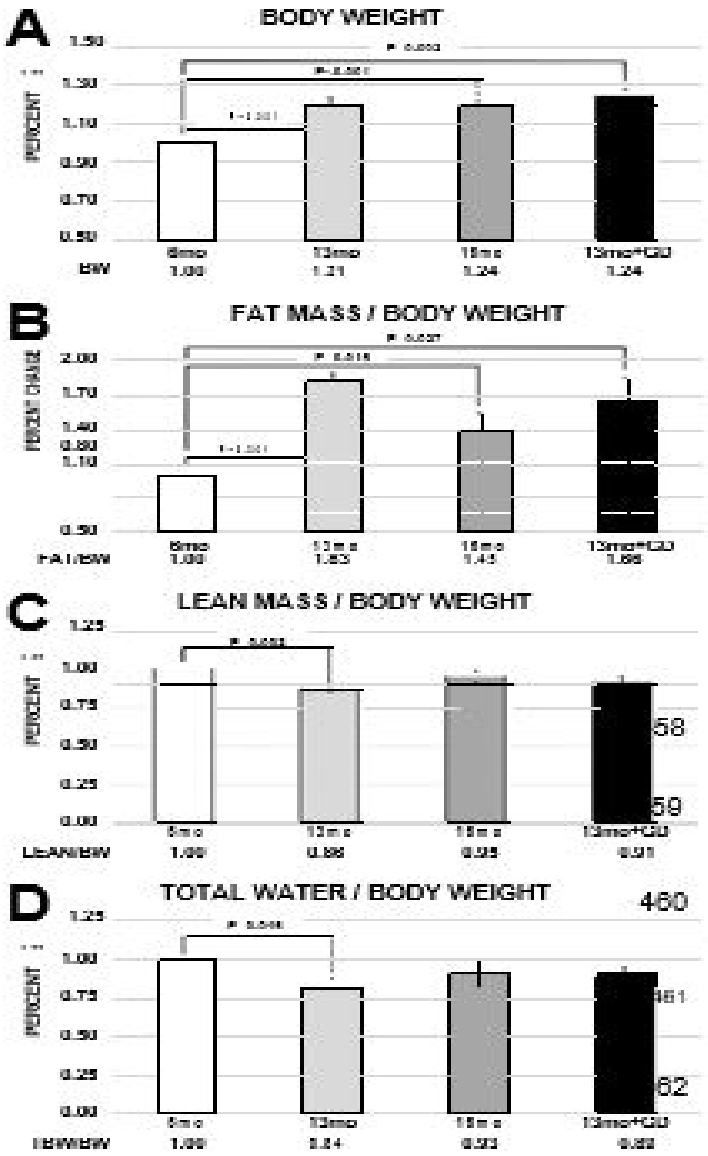


Figure 12(A-D): Body composition. MRI analysis detected significant age-452 associated changes in body composition. A) Body weight was significantly increased with age, but 453 was not influenced by germ cell depletion at 13-months of age. B) Fat mass was significantly increased to 13 months and decreased thereafter. Fat mass454 was modestly decreased with germ cell depletion. C) Lean mass was significantly decreased at 13 455 months, but not at 16 months. Lean mass increased modestly with germ cell depletion at 13 months. D) Total body water was decreased 456 with age and modestly increased with germ cell depletion at 13 months. Values are reported as a percentage of the values in six-457-month-old control mice.

Ex Vivo Anatomic Indices of Health

Heart weight as a fraction of bodyweight was not significantly influenced by age or germ cell depletion (Figure 13). Spleen weight, normalized to bodyweight at death was significantly increased at 13 months of age (51%) with a lesser increase recorded at 16 months of age (23%). Total kidney weight as a fraction of bodyweight was significantly increased in 13-month- old mice (17%), but not in 16-month-old control mice (3%). Germ cell depletion did not significantly influence spleen or kidney weight at 13 months of age.

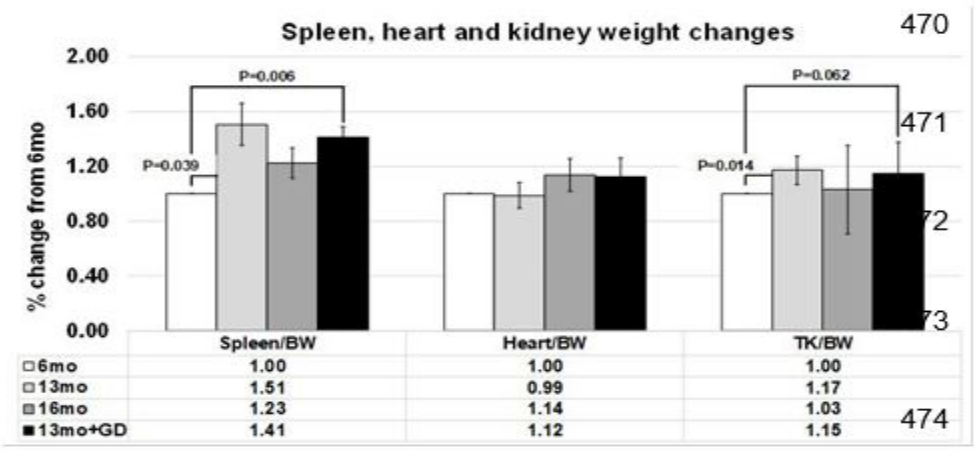


Figure 13: Organ weights. Spleen and kidney weight, but not heart weight was 475 significantly increased at 13 months of age. No significant changes were observed at 16 months of age or with germ cell depletion at 13 months of age. (Total kidney weight-TK; left kidney + right kidney). Organ weights were normalized to Body Weight (BW). Values are reported as a percentage of the values in adult, six-month old control mice.

Discussion

In mammals, young females hold a significant health advantage over males of the same age. With advancing age, in women, reproductive senescence at menopause brings about changes in ovarian signaling that sharply increase susceptibility to cardiovascular and neurodegenerative diseases, diabetes, arthritis and other diseases [4,26] The mechanisms responsible for the abrupt increase in disease triggered by menopause are unclear. A classic view of the initiating cause of menopause is the loss of circulating cyclic ovarian hormones produced by active ovarian germ cells [16]. Accelerated ovarian germ cell activation leads to premature menopause, premature aging and increased disease risks. In previous experiments, transplantation of young, active ovaries mitigated many of these disease risks when used to replace the senescent ovaries in postreproductive mice [12]. Initially, we suspected germ cell hormones in this phenomenon. To test this hypothesis, in the current experiments we chemically depleted ovarian germ cells from prepubertal mice. In this way, treated mice would have no exposure to actively cycling ovarian hormones, but would still be exposed to non-cyclic, non-germ cell hormonal and/or other potential regulatory actions of the germ cell-less ovaries. At 13-months of age, CBA/J mice are reproductively senescent, but have only been without cycling ovarian hormones for a short period (approximately two months). This facilitated obtaining measures of health span from mice that were exposed to cyclic ovarian hormones during a normal reproductive life span and mice without cyclic ovarian hormone exposure, while still avoiding the complications associated with assaying mice at old ages. In the current experiments, assessment of health span was accomplished using *in vivo* measures of neuromuscular, sensory, cognitive, immune, renal and metabolic function and body composition.

Neuromuscular function declines with age [27]. Because the decline in muscle function impacts quality of life, assessment of muscle function represents an appropriate indicator of health span. To assess skeletal muscle function, in the current experiments we chose to use an inverted cling grip test. Grip strength, which decreases with age in mice, can be a good predictor of remaining life span [28,29]. The inverted cling grip test has advantages over standard grip strength and measures overall strength and endurance, like a pull-up test in humans [30].

In our mice, a substantial age-associated loss of grip strength occurred at 13 and 16 months of age. Germ cell depletion significantly exacerbated this loss of grip strength in 13- month old mice. This was unexpected as germ cell depletion increased lean muscle mass by 5% in 13-month old females. Previous work in our lab demonstrated that ovariectomized females (no germ cell input) were heavier than control mice at 13 months of age [15]. Germ cell-depleted mice weighed 3% more than 13-month old

control females, but this should not have been enough weight gain to influence the grip test. Muscle strength can be highly influenced by muscle glycogen. The disturbance in glucose metabolism seen in the germ cell-depleted mice may have played a role in the ability to form/store muscle glycogen in the GD mice.

At 13 months of age, neither germ cell-depleted nor control mice were exposed to cyclic ovarian hormones at the time of evaluation. Previous human studies have demonstrated both a significant increase [31] and decrease [32] in lean body mass due to hormone replacement therapy. These contradictory reports demonstrate that the influence of ovarian hormones on lean mass is likely not direct. Age-related deterioration in sensory systems profoundly diminishes the quality of life for elderly people. There is evidence that the ability to perceive and process sensory stimuli influences life span [33]. The effect of aging on sensory function is an important factor to consider when studying the age-related changes in cognition [34].

Because vision is compromised early in life in CBA/J female mice, we focused on olfactory function as a measure of sensory function changes. Olfactory function plays a critical role in health and behavior [35]. The number of sensory neurons that serve as odorant-selective receptor cells decreases with age [36]. Reduced olfactory function has been linked to a wide range of age-related neurological diseases and impaired olfactory function is a significant predictor of mortality [36,37]. In the current experiments, measures of olfactory perception were divided into two components [38]: odor identification (Buried Pellet Test) and olfactory discrimination (Novel Recognition Block Test).

The buried pellet test measures the food motivation aspect of olfaction, testing the ability of hungry, food restricted mice to detect a piece of sweetened cereal buried under the bedding. The age-associated increase in time to pellet discovery in the current experiments offered strong support of this test as a model for age-associated changes in olfactory function. The lack of influence of germ cell depletion in pellet discovery time supports a minimal role for cyclic ovarian hormones in maintenance of this measure of sensory function.

We next used a Novel Recognition Block Test to assess olfactory discrimination, which did not require food restriction. The block test measured the ability of mice to discriminate between a familiar scent and a novel scent. In the current experiment, with increasing age, all the mice preferred to explore the wooden blocks scented with a familiar home-cage odor rather than a block impregnated with novel odors and in general, spent more time exploring all the blocks than did six-month-old mice. The age-associated increased interest in exploring the blocks is expected in normal adult mice [39] and may be the result of a decrease in the ability to detect odors with age, as demonstrated in our buried pellet test. This decreased ability to detect odors may have decreased avoidance behavior in the aged mice (less time spent avoiding the blocks equates to more time spend exploring them). Germ cell depletion led to a decrease in the time spent sniffing all the blocks, but an increase in the time spent with novel blocks, compared with 13-month old control mice. This observation may suggest a restored ability to detect, but not discriminate between odors due to germ cell depletion. The strong correlation between time spent with familiar blocks and time with novel blocks at all ages/treatments supports the use of caution when interpreting these results.

Cognitive function is deduced by measuring performance in specific behavioral tasks. A burrowing task is qualified as a measure of health span by reflecting a behavior or activity of daily living. Our assessment represents a 'hybrid' test with attributes of the traditional food burrowing, nesting and marble burying tests. However, the marble burying tests have previously been shown not to discriminate between mice with specific accelerated aging phenotypes [40]. As expected, our mice displayed a significant age-dependent decline in burrowing behavior. This behavior was modestly negatively influenced by germ cell depletion. Previous work in our lab has demonstrated that restoration of cyclic ovarian hormones in postreproductive-aged mice positively and significantly restored burrowing behavior toward values seen in younger mice (unpublished observations, Mason). These observations suggest that cognitive function and reproductive function have a synergistic relationship, with further support from the observation that women with premature ovarian failure may also be subjected to early cognitive decline, compared with normally menopausal women [26].

Glucose intolerance increases with age. As a measure of glucose tolerance, we used a standard GTT assay. Aged mice are often intolerant of insulin injections and thus our metabolic testing was limited to the glucose tolerance test in the current experiments. The glucose AUC was moderately negatively influenced by germ cell depletion. The first two values for glucose level after glucose injection (15 min and 30 min) were 6 and 10% higher, respectively than the mean of the other groups combined. However, due to the small number of animals and because we chose to use an IP rather than a PO GTT, the differences between groups did not reach significance. Primary Ovarian Insufficiency (POI) is often associated with ovarian germ cell depletion/dysfunction and the risk of insulin resistance is elevated in young women with POI [5]. In the study by Kulaksizoglu et al. [5], women with POI had increased fasting glucose levels (10%), compared with control women. Fasting glucose was no different in our germ cell-depleted mice, but the exaggerated response to glucose injection at 15 min and 30 min suggests the potential development of a pre-insulin-resistant phenotype. In a previous study [41], VCD-treated C57/B6 mice on a high fat diet were significantly more insulin resistant than cycling controls, but these mice also demonstrated a much

higher baseline glucose than controls, suggesting a difference in sensitivity of the strain used in this study and the CBA/J mice used in current study.

However, both results support the importance of cycling ovarian hormones for metabolic homeostasis. Aging leads to a general decline in protective immunity. The most common, age-associated effects are in T-cell mediated immune function, which contribute to many aspects of defense against viral and microbial infections [42]. There is an age-associated decline in the naive subset of T cells and a consequent increase in memory T cells [43,44]. Having an adequate number of naive T cells is crucial for the immune system to respond to novel pathogens. Adult mice whose immune systems show only moderate changes in T-cell subsets tend to live longer than age-matched siblings that display extensive T-cell subset aging [45].

In the present study, there was a significant age-associated decline in central and peripheral naive T-cell subsets from 6 to 13 and 16 months of age. This naive T-cell decline was not influenced by prepubertal ovarian germ cell depletion in 13-month-old females. Therefore, the loss of cyclic ovarian hormones was likely not critical for maintenance of immune function in female mice.

In the current study, age-associated changes in renal function were far less severe than changes seen with immune function. All 13- and 16-month-old mice displayed decreased plasma levels of many renal panel parameters, suggesting a decrease in renal blood flow with no indication of renal impairment. Creatinine levels decreased with age in our mice. An elevated BUN: Cre due to a low creatinine and a BUN within the reference range is unlikely to be of clinical significance. Because of decreased muscle mass, elderly animals may have an elevated BUN: Cre at baseline [46,47]. The elevated BUN: Cre ratio, where urea was elevated out of proportion to creatinine likely indicated decreased renal blood flow because decreased tubular flow enhances creatinine excretion, while increasing urea reabsorption. The CBA/J strain of mice is known to have a high frequency of renal tubulointerstitial lesions after 12 months of age [48]. However, renal pathology in these mice does not resemble that seen in mice with severe glomerulonephritis, and this strain is not known to develop glomerulonephritis [49]. Peripubertal rats treated with VCD had significantly lower serum creatinine concentrations compared with control rats, but BUN showed no effect of treatment [50]. The decreased creatinine in VCD-treated 13-month old mice may have been due to renal toxicity of the VCD treatment itself and not hormonal influence. However, hormone replacement in postreproductive mice restored cardioprotective influences [13], suggesting a cardiovascular mediated change in renal function.

In 13-month old mice, germ cell depletion decreased the percent fat mass and increased the percent lean mass. Previous work in our laboratory demonstrated that increased ovarian exposure by transplanting new, actively cycling ovaries increased lean mass and decreased fat mass and body weight in 16-month old new ovary recipients. Germ cell depletion also produced an increase (6%) in total body water in 13-month old mice. Generally, total body water decreases with age, with the highest levels present at birth and a precipitous drop thereafter until death [51]. Restoration of total body water by germ cell depletion may suggest restoration of a physiologically younger state. These observations suggest that cyclic ovarian hormones contribute indirectly to regulation of body composition in female mice.

Both spleen and kidney weight were significantly increased in 13-month-old mice, but not in 16-month-old mice. Our 13-month-old control mice were physiological adjustment period, being without ovarian hormones for the first time, but not having had enough time to develop adaptive mechanisms. This period of adjustment may have had an influence on the changes seen in the 13-month-old cohort T-cell subsets and BUN: Cre levels and spleen weights, compared with the 16-month-old mice that have had time to adapt to life without ovarian hormones. However, germ cell depletion did not significantly influence spleen or kidney weight at 13 months of age, suggesting that hormone withdrawal had little influence on organ weights.

Conclusions

Overall, we have shown that depletion of ovarian germ cells and the subsequent life-time loss of cyclic ovarian hormones had mild influences on all health span parameters tested, including a negative influence on neuromuscular, metabolic and renal function and cognitive behavior and a positive influence on sensory function and body composition.

A major limitation of this study was the modest number of animals available in each treatment group. However, even with the small number of animals used, many tests displayed large differences between groups, but often failed to reach statistical significance. In addition, our method of chemical germ cell depletion exposed the mice to a toxin during prepubertal ages and we cannot rule out potential effects of the toxic exposure on the health span parameters measured. These findings provide strong incentive for further investigation of the influence of reproductive function and ovarian germ cells and hormones in the preservation of health in young females and the restoration of health in postreproductive females.

Complete graphic abstract has been shown below in (Figure 14).

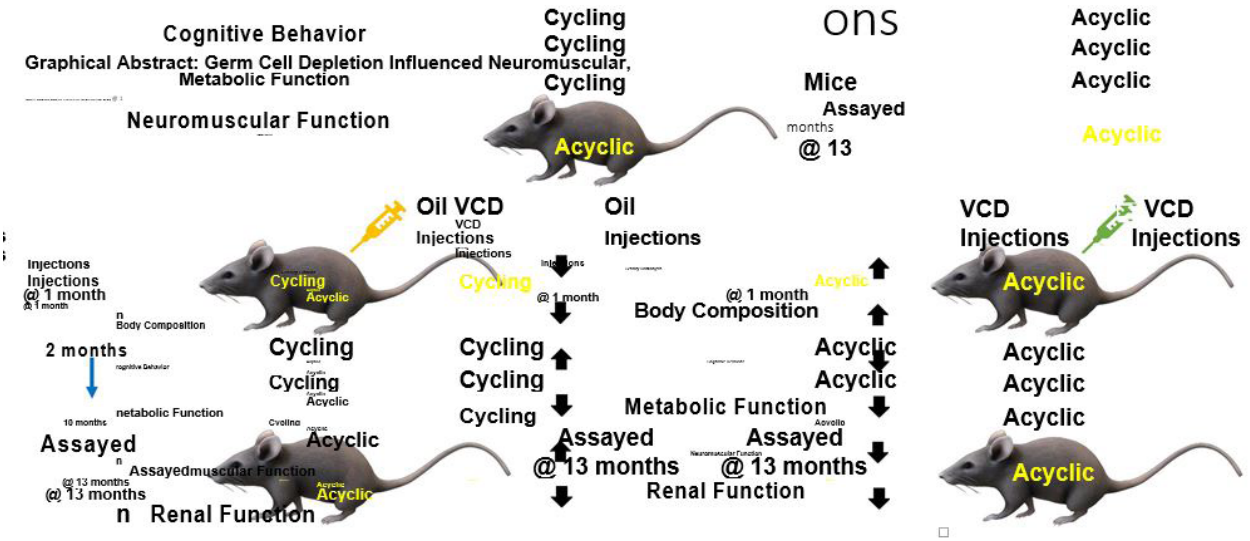


Figure 14: Complete graphic abstract of PostReproductive Female Mice.

Highlights

- Body composition was positively influenced by germ cell depletion.
- Age and germ cell depletion negatively influenced neuromuscular function.
- Renal function was compromised by germ cell depletion.
- Sensory function is influenced strongly by age and mildly by germ cell depletion.
- Cognitive behavior and immune function were not influenced by germ cell depletion.

Acknowledgments

The authors thank Dr. Aaron Olsen and Ms. Lisa DeSoi for help with the mice, Dr. Erik Eide and Mr. Mahdi Nazokkarmaher for help with VCD injections and Ms. Sumira Phatak for guidance with magnetic resonance imaging. Research reported in this publication was supported by Utah State University, School of Veterinary Medicine, Department of Animal, Dairy and Veterinary Sciences Research Initiation Funds.

Authors' declaration of interests

All authors declare that there are no conflicts of interest.

Author Contributions

Conceived and designed the experiments: TLH, KCP, JBM. Performed the experiments: TLH, KCP, JBM. Analyzed the data: TLH, KCP, JBM. Contributed reagents/materials/analysis tools:JBM. Wrote the paper: TLH, KCP, JBM.

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