Title

Antioxidant supplementation partially rescues accelerated ovarian follicle loss, but not oocyte quality, of glutathione-deficient mice†

Journal

Biology of Reproduction, 102(5)

ISSN

0006-3363

Authors

Lim, Jinhwan

Ali, Samiha

Liao, Lisa S

et al.

Publication Date

2020-04-24

DOI

10.1093/biolre/ioaa009

Peer reviewed

Biology of Reproduction, 2020, 102(5), 1065–1079
doi:10.1093/biolre/ioaa009
Research Article
Advance Access Publication Date: 17 January 2020

OXFORD

Research Article

Antioxidant supplementation partially rescues accelerated ovarian follicle loss, but not oocyte quality, of glutathione-deficient mice†

Jinhwan Lim[1], Samiha Ali[1], Lisa S. Liao[1], Emily S. Nguyen[1], Laura Ortiz[1], Samantha Reshel[2] and Ulrike Luderer[1,2,3,*]

[1]Department of Medicine, University of California, Irvine, California, USA, [2]Department of Developmental and Cell Biology, University of California, Irvine, California, USA and [3]Program in Public Health, University of California, Irvine, California, USA

***Correspondence:** Center for Occupational and Environmental Health, 100 Theory Drive, Suite 100, Irvine, CA 92617, USA. Tel: +1 9498248641; E-mail: uluderer@uci.edu;

†Grant Support: Research reported in this publication was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health (NIH) under Award Number R01ES020454, by a NIH Office of Dietary Supplements Supplement to R01ES020454 (to UL) and by University of California Irvine Summer Undergraduate Research Program fellowships (to ESN and SA).

Received 7 August 2019; Revised 16 December 2019; Editorial Decision 13 January 2020; Accepted 13 January 2020

Abstract

The tripeptide thiol antioxidant glutathione (GSH) has multiple physiological functions. Female mice lacking the modifier subunit of glutamate cysteine ligase (GCLM), the rate-limiting enzyme in GSH synthesis, have decreased GSH concentrations, ovarian oxidative stress, preimplantation embryonic mortality, and accelerated age-related decline in ovarian follicles. We hypothesized that supplementation with thiol antioxidants, *N*-acetyl cysteine (NAC), or α-lipoic acid (ALA) will rescue this phenotype. *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ females received 0 or 80 mM NAC in drinking water from postnatal day (PND) 21–30; follicle growth was induced with equine chorionic gonadotropin (eCG) on PND 27, followed by an ovulatory dose of human CG and mating with a wild type male on PND 29 and zygote harvest 20 h after hCG. *N*-acetyl cysteine supplementation failed to rescue the low rate of second pronucleus formation in zygotes from *Gclm*$^{-/-}$ versus *Gclm*$^{+/+}$ females. In the second study, *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ females received diet containing 0, 150, or 600 mg/kg ALA beginning at weaning and were mated with wild type males from 8 to 20 weeks of age. α-Lipoic acid failed to rescue the decreased offspring production of *Gclm*$^{-/-}$ females. However, 150 mg/kg diet ALA partially rescued the accelerated decline in primordial follicles, as well as the increased recruitment of follicles into the growing pool and the increased percentages of follicles with γH2AX positive oocytes or granulosa cells of *Gclm*$^{-/-}$ females. We conclude that ovarian oxidative stress is the cause of accelerated primordial follicle decline, while GSH deficiency per se may be responsible for preimplantation embryonic mortality in *Gclm*$^{-/-}$ females.

Summary sentence

Supplementation with the antioxidant alpha-lipoic acid partially rescued accelerated age-related ovarian follicle depletion, but did not decrease offspring production, in glutathione-deficient *Gclm* null mice.

Key words: ovary, follicle, oocyte, oxidative stress, female infertility, premature ovarian failure

Introduction

Premature ovarian failure (POF, also called primary ovarian insufficiency) is manifested by accelerated depletion of the ovarian follicle pool and poor oocyte quality. Clinically, POF is defined as cessation of ovarian function before the age of 40, while the normal age of menopause is 50 ± 4 years [1, 2]. Premature ovarian failure affects 1% of women. Although abnormalities of the X chromosome, various rare mutations, exposure to chemotherapy, and ionizing radiation are known causes of POF, the cause remains unknown in 90% of cases [2–4]. Oxidative stress has been implicated in POF caused by exposure to toxicants and ionizing radiation [5–7].

The tripeptide glutathione (GSH, γ-glutamyl cysteinyl glycine) is the most abundant intracellular nonprotein thiol, with intracellular concentrations in the millimolar range. Glutathione has numerous intracellular functions, including antioxidant defenses, reduction of disulfide bonds, and S-glutathionylation as a post-translational protein modification [8–12]. Glutathione can scavenge free radicals directly, participate in the reduction of hydrogen peroxide and lipid peroxides as a cofactor for glutathione peroxidases and peroxiredoxin, and detoxify electrophilic toxicants as a cofactor for glutathione transferase [8, 9, 13]. Glutathione is synthesized in two ATP-dependent reactions. The first rate-limiting reaction is catalyzed by glutamate cysteine ligase (GCL), a heterodimer composed of a catalytic (GCLC), and a modifier (GCLM) subunit [14, 15]. Glutamate cysteine ligase catalytic subunit is responsible for the catalytic activity of the enzyme, while binding of GCLM to GCLC increases GCL enzymatic activity [14, 15]. *Gclc*$^{-/-}$ mice die during embryonic development [16], whereas *Gclm*$^{-/-}$ mice survive to advanced age, despite greatly decreased tissue levels of GSH ([17, 18] and our unpublished observations). Our prior studies showed that *Gclm*$^{-/-}$ mice have low ovarian GSH concentrations, chronic ovarian oxidative stress, poor oocyte quality resulting in delayed male pronucleus formation in zygotes and early preimplantation embryonic mortality, and accelerated age-related decline in ovarian follicle numbers [19, 20]. The *Gclm*$^{-/-}$ mouse model thus recapitulates the decreased oocyte quality and early depletion of the ovarian reserve, which are key characteristics of idiopathic POF in women. Moreover, these prior findings support a role for ovarian oxidative stress as a driver of POF.

N-acetyl cysteine (NAC) increases GSH synthesis by providing a source of cysteine, the least abundant amino acid constituent of GSH. Glutathione itself is not taken up by cells [21, 22]. *N*-acetyl cysteine is orally bioavailable, non-toxic, and has been shown to be protective against acetaminophen-induced hepatotoxicity, which is caused by depletion of GSH and induction of mitochondrial oxidative stress by the reactive metabolite *N*-acetyl-*p*-benzoquinone imine [23]. *N*-acetyl cysteine is used clinically to treat acetaminophen hepatotoxicity in humans [24]. *N*-acetyl cysteine administered at 40 mM in the drinking water has been reported to increase pulmonary and blood, but not hepatic or brain, GSH levels and to protect against CNS oxygen toxicity in rats [25]. *N*-acetyl cysteine at 20 mM in drinking water administered to pregnant dams increased GSH levels in control mouse fetuses, but not in fetuses simultaneously exposed to buthionine sulfoximine, a specific inhibitor of GCL that depletes GSH by inhibiting its synthesis [26]. *N*-acetyl cysteine treatment did however protect against DNA damage caused by buthionine sulfoximine-induced GSH depletion in the fetuses, despite no increase in GSH concentrations [26]. Other studies also support antioxidant effects of NAC that are independent of its ability to enhance GSH synthesis [27, 28].

α-Lipoic acid (ALA) is a dithiol that is generated enzymatically in mitochondria from octanoic acid, where it is a necessary cofactor for α-ketoacid dehydrogenases [29]. α-Lipoic acid is also absorbed from dietary sources and is widely available as a nutritional supplement [29, 30]. In vitro, ALA is a direct acting antioxidant and metal chelator; however, concentrations required for these effects may not be achievable in vivo [29]. In vivo, ALA increases ascorbate and cysteine uptake from plasma and induces expression of numerous antioxidant genes, including *Gclc* and *Gclm,* by increasing de novo translation of the transcription factor Nuclear Factor, Erythroid 2 like 2 (NFE2L2, also known as NRF2) [29, 31, 32]. Dietary supplementation with ALA has been reported to protect against several conditions in which oxidative stress is thought to play a role, including diabetic neuropathy and insulin resistance, in humans [33–35]. Supplementation with dietary ALA protected C57BL/6 J mice against age-related hearing loss, which is caused by upregulation of proapoptotic genes in the cochlea by mitochondrial ROS [36]. Supplementation with intraperitoneal injections of ALA reportedly protected mice against depletion of the ovarian reserve by treatment with the anticancer drug methotrexate [37]. α-Lipoic acid supplementation decreased age-related mitochondrial oxidative damage and GSSG concentrations and increased mitochondrial GSH concentrations in the brains of rats [38]. Supplementation of media with ALA, NAC, and acetyl-L-carnitine alone or in combination during culture of mouse zygotes has been reported to improve development to the blastocyst stage [39].

If the hypothesis that oxidative stress is driving either the early embryonic mortality and/or the accelerated age-related follicle decline in *Gclm*$^{-/-}$ mice is correct, then providing these mice with an additional means of protection against oxidative stress should rescue these phenotypes. We first tested the efficacy of short-term supplementation with NAC during the later stages of oocyte maturation to ameliorate the reduced offspring production due to early embryonic mortality in *Gclm*$^{-/-}$ mice. We then tested whether long-term supplementation with ALA could rescue the decreased offspring production and/or slow the accelerated decline in ovarian follicles observed in *Gclm*$^{-/-}$ mice.

Materials and methods

Reagents

N-acetyl cysteine (# A7250) and ALA (# 62320) were obtained from Sigma Aldrich. α-Lipoic acid was sent to Bio-Serv (Flemington, NJ) to formulate three different diets: (1) control diet was Rodent Diet,

AIN-93 M ½″ pellets (# F3155), (2) AIN-93 M diet supplemented with 150 mg/kg ALA, and (3) AIN-93 M diet supplemented with 600 mg/kg ALA. The caloric profile of the rodent diet was a total of 3.58 kcal/g, with 0.51 kcal/g as protein, 0.37 kcal/g as fat, and 2.70 kcal/g as carbohydrate. All other reagents and chemicals were obtained from Sigma Aldrich or Fisher Scientific unless otherwise noted.

Animals

Gclm$^{-/-}$ mice were generated by disrupting the *Gclm* gene by replacing exon 1 with a β-galactosidase/neomycin phosphotransferase fusion gene and backcrossing onto a C57BL/6 J genetic background [18, 40]. Mice for these experiments were bred by crossing *Gclm*$^{+/-}$ males and females in our colony. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility on a 14:10 h light-dark cycle, with temperature maintained at 69–75 °F. Mice had free access to deionized water and soy-free laboratory chow (Harlan Teklad 2919), except when placed on experimental diets described below. The experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals [41] and were approved by the Institutional Animal Care and Use Committee at the University of California Irvine.

Experimental protocols

For all experiments, *Gclm*$^{+/+}$ and *Gclm*$^{-/-}$ females were randomly assigned to treatment groups using a random number table, avoiding assignment of littermates of the same genotype to the same experimental group. All endpoints were analyzed by investigators blind to experimental group.

Experiment 1: Effect of short-term NAC supplementation during later stages of oocyte maturation on fertilization and zygote development. *Gclm* $^{+/+}$ and *Gclm*$^{-/-}$ mice were given ad libitum access to NAC-supplemented drinking water (80 mM, pH adjusted to 6.0 with fresh NaOH) or unsupplemented water beginning at 21 days of age, then injected with equine chorionic gonadotropin (eCG) to induce follicle development at 27 days of age, followed by injection of hCG to stimulate ovulation (while continuing to receive NAC in the drinking water) 46 h later. The NAC concentration was chosen to be higher than concentrations found to be effective in previous studies [25, 26] because we hypothesized that the *Gclm*$^{-/-}$ mice would require higher concentrations to use NAC directly as an antioxidant due to limited ability to upregulate GSH synthesis despite increased cysteine. *N*-acetyl cysteine-supplemented drinking water was prepared fresh every 2 days [25]. Water consumption and body weight were monitored throughout the study. Females were mated with wild type males the evening of the day of hCG administration. Females were euthanized by CO_2 inhalation, and zygotes were collected from the oviducts 20 h after hCG, as previously described [19]. Numbers of oocytes without evidence of fertilization, oocytes with single sperm or multiple sperm without pronuclei, zygotes with one pronucleus, zygotes with two pronuclei, and fragmented oocytes were counted using DIC microscopy with a Zeiss Axiovert microscope.

Experiment 2: Effect of long-term ALA supplementation on fertility and ovarian follicle numbers. In a preliminary experiment, we found no differences in the amounts of ALA-supplemented diet consumed compared with control diet (data not shown). Therefore, we concluded that pair-feeding was not necessary. *Gclm*$^{+/+}$ and *Gclm*$^{-/-}$ females were fed control AIN-93M chow or AIN-93M chow supplemented with 150 or 600 mg/kg chow of the antioxidant ALA from postnatal day (PND) 21 until euthanasia at approximately 5.5 months of age (N = 8/group for ovarian endpoints, except *Gclm*$^{+/+}$, 600 mg/kg group N = 7; one female removed because of incorrect genotype assignment). Throughout this experiment, body weights and feed consumed were measured biweekly.

From 2 months of age, each female was individually housed with a proven breeder wild type male. Females were checked each morning for 12 weeks (84 days) for abdominal distention indicative of pregnancy and for births. When a birth was observed, the number of live and dead pups, and sex and weight of live pups were recorded. Newborn pups were euthanized by hypothermia followed by decapitation. At the end of 12 weeks of the breeding study or after the last litter was born if that occurred after 12 weeks, vaginal cytology was performed for at least 2 weeks to monitor estrous cycles, and mice were euthanized on the day of metestrus of the cycle by CO_2 inhalation. One ovary was fixed in Bouin's solution for follicle counts and the other was fixed in 4% paraformaldehyde (PFA) for immunostaining. For the breeding study endpoints, one female was removed from the *Gclm*$^{+/+}$, 600 mg/kg group (N = 6 for breeding endpoints) and one from the *Gclm*$^{-/-}$, 150 mg/kg ALA group (N = 7 for breeding endpoints) because they were inadvertently not checked for birth for several days so pup number and weights at birth could not be ascertained.

Experiments 3 and 4: Effects of NAC or ALA supplementation on ovarian GSH concentrations. To determine if NAC administration increases ovarian GSH concentrations in *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ mice, 2-month old *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ female mice were given ad libitum access to NAC-supplemented (80 mM) or unsupplemented drinking water for 7 days, timed based on the mouse's estrous cycle so that they were euthanized on the day of metestrus. Water consumption and body weight were monitored throughout the study. Mice were euthanized by CO_2 inhalation, and ovaries plus attached oviducts were homogenized in six times volume in microlitre per milligram tissue weight 20 mM Tris, 1 mM EDTA, 250 mM sucrose, 2 mM l-serine, 20 mM boric acid (TES-SB) for GSH and GSSG assay as we have previously described [42, 43]. Briefly, after removal of a 10 µL aliquot of homogenate for protein assay, remaining homogenates were acidified with one quarter volume 5% sulfosalicylic acid; the homogenates were processed directly for total GSH assays (GSH plus GSSG). For GSSG assay, GSH in the homogenates was first conjugated with 2-vinylpyridine and extracted with chloroform, and the aqueous phase was then assayed. Triplicates of samples or standards were combined with 33 µL metal free water and incubated for 10 min at 30 °C. The samples were mixed with 140 µL of 0.3 mM NADPH, 20 µL of 6 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), and 2 µL of 50 units/mL GSH reductase. The rate of thiobis (2-nitrobenzoic acid) (TNB) formation from DTNB is proportional to the total GSH concentration in each sample. Thiobis (2-nitrobenzoic acid) formation was monitored by measuring the absorbance at 412 nm for 5 min every 10 s using a VersaMax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentrations of total GSH in the samples were calculated from a standard curve generated from the slopes of the standards.

To determine if ALA supplementation increases ovarian GSH concentrations, 21-day old *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ female mice were given ad libitum access to control or ALA supplemented diets. Beginning at 8 week of age, vaginal cytology was performed daily for at least two estrous cycles, and mice were euthanized by CO_2

on the day of metestrus. Both ovaries were processed for total GSH assay as above.

Estrous cycling

For evaluation of estrous cycling, mice were individually housed, and vaginal lavage was performed every morning using 0.9% NaCl for at least 14 days. Cells in the lavage fluid were examined by light microscopy immediately after collection, and the predominant cell types present in the fluid were recorded as previously described [44].

Ovarian follicle counts

The ovaries from ALA-supplemented or control mice were fixed in Bouin's solution overnight at 4 °C and embedded as a group in blocks (five ovaries per block) of glycol methacrylate resin (Technovit 8100; Heraeus Kulzer GmbH, Wehrheim, Germany) to maximize sectioning and counting efficiency. Each block was serially sectioned at 20 µm followed by hematoxylin and eosin staining. Every fifth section of each ovary was used to estimate the total number of primordial and primary follicles using stereological software, Stereo Investigator 2017 (MBF Bioscience Inc., Williston, VT, USA) in conjunction with an Olympus BX-40 microscope fitted with 4× PLAN FLUO and 60× (N_A = 1.4) U PLAN FLUO oil immersion objectives and a motorized stage controlled via the software. A 2D counting frame (45 µm × 45 µm; 2025 µm^2) was generated and superimposed over each ovary section by the Optical Fractionator probe for systematic random sampling and then shifted progressively along the XY-axis in a sampling grid that subdivided the sections into 90 µm × 90 µm (8100 µm^2) squares. Oocytes with nucleus of each follicle were counted if they fell entirely or partially within the inclusion boundaries of the counting frame and if they came into sharp focus within the middle 8 µm of the section (*Z*-axis). The upper (2 µm) and lower (2 µm) portions of the sections were excluded from counting to avoid sectioning artifact. Raw counts from each ovary were then converted to an estimate of the total number in the entire ovary by multiplying times the reciprocal of the counting fractions: raw count × section interval (5) × grid frame area/counting frame area (8100/2025) × average section thickness/counting frame height × 12/8 [45, 46]. Secondary and antral follicles were counted using an Olympus BX-60 microscope by following through every section of the ovary to avoid double counting. Follicles were classified as primordial, primary, secondary, and antral and as healthy or atretic as previously described [20].

Immunohistochemistry

All PFA fixed ovaries from the ALA experiment were cryoprotected in 30% sucrose in PBS at 4 °C, embedded in optimal cutting temperature compound, and stored at −80 °C before being serially sectioned using a cryostat at a thickness of 10 µm. Immunohistochemistry was performed using the Vectastain ABC kit (PK-4001; Vector Laboratories, Burlingame, CA, USA). Briefly, sections were thawed and heated for 15 min at 95 °C in a 10 mM citrate buffer (pH 6.0). The primary antibodies—rabbit anti-cleaved caspase-3 Asp 175 (1:100; Cell Signaling #9664, Beverly, MA, USA), rabbit anti-Ki67 (1:500; Abcam #15580, Cambridge, MA, USA), and rabbit anti-γH2AX (1:200; Cell Signaling #9718)—were detected using biotinylated goat anti-rabbit secondary antibody in 5% normal goat serum. All immunostaining procedures included avidin/biotin and 3% hydrogen peroxide blocking steps. Peroxidase activity was visualized using 3,3′-diaminobenzidine as substrate (Roche). Sections were counterstained with hematoxylin. The following negative controls were included in every experiment: secondary antibody without primary antibody; primary antibody without secondary antibody; and primary antibody replaced by rabbit IgG with secondary antibody. Both negative and positive follicles for each marker were counted and quantities expressed as fractions were used for statistical analyses.

The numbers of Ki67, cleaved caspase-3, and phosphorylated histone 2AX (γH2AX)-positive and -negative follicles or oocytes were counted in one section per slide for 3 or 4 slides per endpoint distributed throughout the ovary by an investigator (JL, LL, and SA) blind to *Gclm* genotype and ALA treatment using an Olympus BX-60 microscope with 10, 20, and 40× U PLAN FLUO objectives equipped with a Retiga 2000R cooled CCD digital camera system with Q-Capture Pro software. The percentages of positive primordial and primary follicles (containing one or more positive granulosa cells per largest cross-section or containing positive oocytes) and secondary and antral follicles (containing three or more positive granulosa cells per largest cross-section or containing positive oocytes) were calculated and used for data presentation and analysis.

Statistical analysis

All values are presented as mean ± SEM in figures and tables. Two-way ANOVA was used to test the effects of genotype and antioxidant supplementation on continuous endpoints (follicle numbers, number of oocytes ovulated, and GSH concentrations). Quantities expressed as fractions were subjected to arcsine square root transformation prior to ANOVA [47] or analyzed by nonparametric Kruskal–Wallis test followed by Mann–Whitney tests for intergroup comparisons. If statistically significant effects or interactions were observed in the two-way ANOVA, we performed one-way ANOVA followed by LSD test for intergroup comparisons. Statistical analyses were performed using SPSS 25 for Mac OS X (IBM Software).

Results

N-acetyl cysteine supplementation does not rescue zygote development in Gclm$^{-/-}$ females

This experiment was designed to test whether short-term treatment with the thiol antioxidant NAC during the later stages of oocyte maturation could rescue the decreased ability of fertilized eggs from *Gclm*$^{-/-}$ females to reach the two pronucleus stage by 0.5 days post-coitum [19]. Consistent with our prior findings [19], *Gclm*$^{+/+}$ and *Gclm*$^{-/-}$ females ovulated similar numbers of oocytes in response to exogenous gonadotropin stimulation, but at 0.5 days post coitum, *Gclm*$^{-/-}$ females had significantly higher percentages of zygotes with one pronucleus (P = 0.010, effect of genotype) and significantly lower percentages of zygotes with two pronuclei (P < 0.001, effect of genotype) compared with *Gclm*$^{+/+}$ females (Table 1). Supplementation with 80 mM NAC in drinking water for 1 week prior to superovulation failed to rescue the low rate of second pronucleus formation in zygotes from *Gclm*$^{-/-}$ females compared with zygotes from *Gclm*$^{+/+}$ females (Table 1). Both *Gclm*$^{+/+}$ and *Gclm*$^{-/-}$ females supplemented with NAC tended to ovulate fewer oocytes than females drinking unsupplemented water (P = 0.050, effect of NAC).

ALA supplementation does not rescue the decreased offspring production of Gclm$^{-/-}$ females

To test whether long-term antioxidant supplementation could rescue the decreased litter size due to preimplantation embryonic mortality in *Gclm*$^{-/-}$ mice, we chose the thiol antioxidant ALA because it can

Table 1. Effects of NAC supplementation on ovulation and early embryonic development

	0 mM NAC		80 mM NAC	
	Gclm$^{+/+}$ ($N = 5$)	*Gclm*$^{-/-}$ ($N = 5$)	*Gclm*$^{+/+}$ ($N = 8$)	*Gclm*$^{-/-}$ ($N = 5$)
# oocytes ovulated[a]	54.8 ± 4.2	58.4 ± 2.7	40.4 ± 10.7	32.0 ± 12.3
% unfertilized or fragmented[b]	19.7 ± 5.4	29.5 ± 3.4	59.0 ± 9.0	26.2 ± 9.9
% fertilized[c]	80.3 ± 5.4	70.5 ± 3.4	41.0 ± 9.0	73.8 ± 9.9
% zygotes, 1 pronucleus[d]	1.1 ± 0.8	34.2 ± 4.5	9.5 ± 6.6	42.1 ± 15.2
% zygotes, 2 pronuclei[e]	53.5 ± 11.8	7.2 ± 3.6	21.6 ± 9.7	9.6 ± 6.3

[a]NS, effects of genotype and NAC.
[b]$P = 0.099$, effect of NAC; $P = 0.017$, genotype × NAC.
[c]$P = 0.099$, effect of NAC; $P = 0.017$, genotype × NAC.
[d]$P < 0.001$, effect of genotype.
[e]$P = 0.010$, effect of genotype.

be administered via the diet. Dietary supplementation was started at weaning when the mice were 21 days old and continued throughout a 12-week continuous breeding assay that began when the mice were 2 months old. We did not observe evidence of beneficial effects of dietary ALA supplementation on cumulative numbers of litters or offspring in *Gclm*$^{-/-}$ females, and we observed evidence of an adverse effect of the 600 mg/kg ALA-supplemented diet on fertility in *Gclm*$^{+/+}$ females (Figure 1A and B). There were small, statistically significant effects of *Gclm* genotype on the cumulative number of litters produced by *Gclm*$^{-/-}$ compared with *Gclm*$^{+/+}$ females at 28, 56, and 84 days of continuous breeding ($P \leq 0.02$, effect of genotype, two-way ANOVA), with the *Gclm*$^{-/-}$ females in the control and 150 mg/kg ALA diet groups having 28 and 18% fewer litters at 84 days compared with *Gclm*$^{+/+}$ females in the same diet group (Figure 1A). There was also a statistically significant interaction between genotype and diet at 84 days ($P = 0.012$), with the number of litters tending to increase with increasing dietary ALA in the *Gclm*$^{-/-}$ females, but decreasing with the 600 mg/kg ALA diet in the *Gclm*$^{+/+}$ females.

Gclm$^{-/-}$ females produced significantly fewer offspring at each time point ($P < 0.001$, effect of genotype; Figure 1B). There was no statistically significant effect of diet on cumulative offspring at any time point, but the interaction between genotype and diet approached significance at 84 days, with decreased numbers of offspring in the *Gclm*$^{+/+}$, 600 mg/kg ALA group compared with *Gclm*$^{+/+}$ controls ($P = 0.06$).

The percentage of dead pups and the body weights of male and female pups on the day of birth did not differ significantly with genotype or diet (data not shown).

No Gclm genotype- or ALA diet-related differences in estrous cycling

At the end of the breeding study, dietary ALA supplementation continued while vaginal cytology was conducted for at least 14 days to assess estrous cycling. Most females of both *Gclm* genotypes on the various ALA diets—control, 150 mg/kg, and 600 mg/kg—had regular 4- to 5-day estrous cycles (Table 2). The percentages of days of metestrus or diestrus, characterized by leukocytic vaginal cytology, proestrus, characterized by nucleated and cornified cells, and estrus, characterized by cornified cells, were similar between genotypes and diets ($P > 0.05$ effects of genotype and diet on all estrous cycle endpoints; Table 2).

ALA supplementation attenuated the accelerated age-related decline in ovarian follicles in Gclm$^{-/-}$ mice

Mice from the breeding study were euthanized on metestrus of the estrous cycle, and ovarian follicles were enumerated. There was a statistically significant effect of *Gclm* genotype on total number of healthy follicles of all stages of development ($P = 0.002$, effect of genotype, $P = 0.203$, effect of diet; $P = 0.748$ genotype by diet interaction two-way ANOVA; Figure 2A). Intergroup comparisons showed that *Gclm*$^{-/-}$ mice fed the control diet had significantly fewer follicles than *Gclm*$^{+/+}$ mice on the control diet ($P = 0.020$), while total follicle counts did not differ significantly between *Gclm*$^{-/-}$ mice on the 150 mg/kg ALA diet and *Gclm*$^{+/+}$ mice on any of the diets ($P \geq 0.136$) or between *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ mice on the 600 mg/kg ALA diet ($P = 0.065$). Total follicle counts in *Gclm*$^{-/-}$ mice on the 600 mg/kg diet were significantly lower than total follicle counts in *Gclm*$^{+/+}$ mice on the control ($P = 0.005$) or 150 mg/kg ($P = 0.004$) diets.

There were statistically significant effects of *Gclm* genotype on numbers of primordial follicles ($P < 0.001$, effect of genotype; $P = 0.378$, effect of diet; $P = 0.748$ genotype by diet interaction two-way ANOVA; Figure 2B) and primary follicles ($P = 0.037$, effect of genotype; $P = 0.149$, effect of diet; and $P = 0.762$ genotype by diet interaction; Figure 2C) per ovary, with *Gclm*$^{-/-}$ females having fewer primordial and primary follicles than *Gclm*$^{+/+}$ females. Intergroup comparisons revealed that *Gclm*$^{-/-}$ mice fed control diet or 600 mg/kg ALA diet had 68% and 61% fewer primordial follicles, respectively, than *Gclm*$^{+/+}$ females fed the same diets ($P = 0.009$ and $P = 0.049$), while *Gclm*$^{-/-}$ mice fed 150 mg/kg ALA chow had only 45% fewer primordial follicles than *Gclm*$^{+/+}$ females fed the same diet ($P = 0.061$) or the control diet ($P = 0.067$) and only 29% fewer follicles than *Gclm*$^{+/+}$ on the 600 mg/kg diet ($P = 0.347$; Figure 2B). *Gclm*$^{-/-}$ mice fed the 600 mg/kg ALA diet had significantly fewer primordial follicles than *Gclm*$^{+/+}$ mice on the control ($P = 0.005$) and 150 mg/kg diets ($P = 0.004$). Intergroup comparisons showed that the ovaries of *Gclm*$^{-/-}$ mice fed the 600 mg/kg ALA diet had significantly fewer primary follicles than ovaries of *Gclm*$^{+/+}$ mice fed the control or 150 mg/kg ALA diets ($P = 0.015$, $P = 0.017$, respectively; Figure 2C); no other intergroup comparisons were statistically significant for primary follicle numbers. There were no statistically significant effects of *Gclm* genotype or dietary ALA supplementation on numbers of secondary and antral follicles (Figure 2D and E).

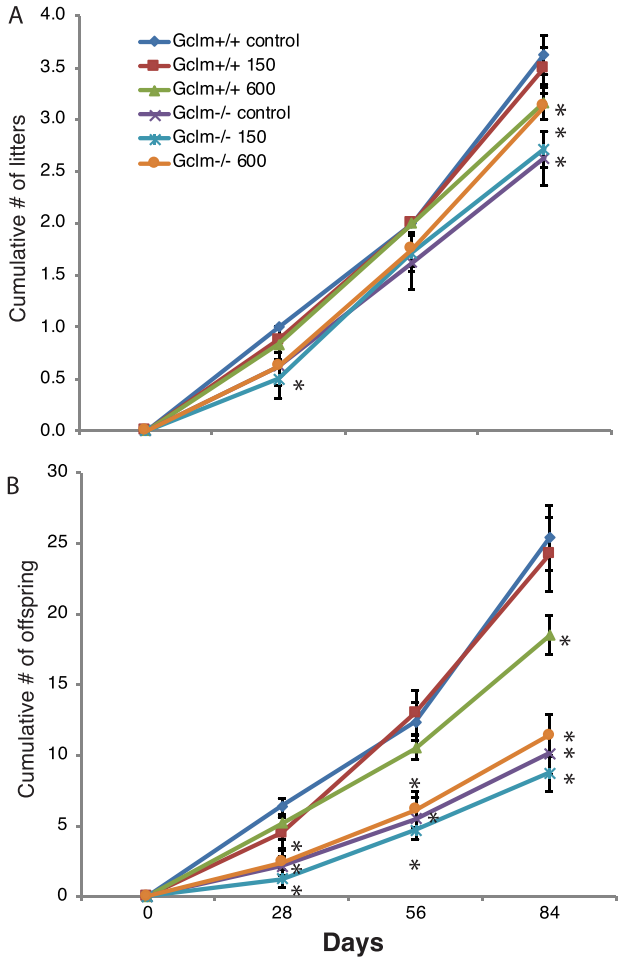

Figure 1. Dietary ALA-supplementation did not rescue the decreased fertility of *Gclm*$^{-/-}$ females. Female *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ mice were fed diets supplemented with 0, 150, or 600 mg/kg ALA from 3 weeks of age until euthanasia. Females were bred continuously with a wild type male from 2 months of age and litter and offspring production were assessed for 12 weeks as described in Materials and methods. (A) Litter production was decreased in *Gclm*$^{-/-}$ females, with a statistically significant effect of genotype at 28, 56, and 84 days ($P \leq 0.02$). The diet by genotype interaction was statistically significant at 84 days ($P = 0.012$), due to lower offspring production in *Gclm*$^{+/+}$ females fed the 600 mg/kg ALA diet at 84 days. (B) Offspring production was significantly less in all *Gclm*$^{-/-}$ groups compared with *Gclm*$^{+/+}$ controls ($P < 0.001$, effect of genotype at all-time points). There was a significant diet by genotype interaction at 84 days ($P = 0.012$), with *Gclm*$^{+/+}$ females fed the 600 mg/kg ALA diet having significantly fewer offspring than *Gclm*$^{+/+}$ controls. The graphs show means ± SEM cumulative number of litters or offspring produced by females of the indicated *Gclm* genotypes and ALA diet at 28, 56, and 84 days of continuous breeding. $N = 8$/group, except $N = 6$ *Gclm*$^{+/+}$, 600 mg/kg ALA group; $N = 7$ *Gclm*$^{-/-}$ 150 mg/kg ALA group). $^{*}P < 0.05$, compared with *Gclm*$^{+/+}$ controls at the same time point.

Figure 3 shows the fraction of primordial (Figure 3A) and primary (Figure 3B) follicles out of the total number of healthy follicles. Primordial follicles make up a smaller fraction ($P = 0.007$, effect of genotype; $P = 0.686$, effect of diet; $P = 0.279$, diet × genotype interaction) and primary follicles make up a non-significantly larger fraction ($P = 0.056$, effect of genotype; $P = 0.772$, effect of diet; $P = 0.725$, diet × genotype interaction) of all healthy follicles in *Gclm*$^{-/-}$ mice compared with *Gclm*$^{+/+}$ mice on the control diet. The fraction of primordial follicles increased from 0.27 in *Gclm*$^{-/-}$ mice fed control diet to 0.39 in *Gclm*$^{-/-}$ mice fed 150 mg/kg ALA diet. Intergroup comparisons revealed that the fraction of primordial follicles in *Gclm*$^{+/+}$ mice on the control diet or the 600 mg/kg diet was greater than the fraction of primordial follicles in *Gclm*$^{-/-}$ mice on the control diet ($P = 0.010$ and $P = 0.012$, respectively), while the fraction of primordial follicles in the *Gclm*$^{-/-}$ mice on the 150 mg/kg diet did not differ from those of *Gclm*$^{+/+}$ mice on any of the three diets (≥0.125). The fraction of primary follicles tended to be higher in *Gclm*$^{-/-}$ mice compared with *Gclm*$^{+/+}$ mice overall, with minimal effect of diet ($P = 0.056$, effect of genotype; $P = 0.772$, effect of diet; $P = 0.725$ genotype by diet interaction). These data provide further support that supplementation with 150 mg ALA/kg diet shifted follicle dynamics in *Gclm*$^{-/-}$ mice toward those observed in *Gclm*$^{+/+}$ mice.

ALA supplementation prevented the accelerated recruitment of primordial follicles in Gclm$^{-/-}$ mice

As quiescent primordial follicles are recruited into the growing pool, the granulosa cells begin to undergo mitosis [48]. To test whether the apparent beneficial effect of 150 mg/kg ALA diet on ovarian follicle numbers in *Gclm*$^{-/-}$ mice could be due to slowed recruitment of follicles into the growing pool, we used Ki67, a specific marker of mitosis [49], to identify recruited follicles based on granulosa cell proliferation. The average percentage of primordial follicles with Ki67-positive granulosa cells was below 15% in all groups and did not differ significantly by *Gclm* genotype or dietary ALA concentration (Figure 4C). In contrast, the percentages of primary follicles with Ki67-positive granulosa cells varied significantly with dietary ALA concentration, and *Gclm* genotype by diet interaction ($P = 0.033$, $P = 0.018$, respectively, by two-way ANOVA). Intergroup comparisons showed that, consistent with our previous observations [20], the percentage of Ki67-positive primary follicles was significantly higher in *Gclm*$^{-/-}$ ovaries from mice fed the 0 mg/kg ALA diet than in *Gclm*$^{+/+}$ ovaries from mice fed the same diet ($P = 0.016$; Figure 4D). In contrast, the percentage of Ki67-positive primary follicles in *Gclm*$^{-/-}$ mice fed 150 mg/kg ALA diet did not differ from *Gclm*$^{+/+}$ females fed the same diet ($P = 0.232$) or the 0 mg/kg ALA diet ($P = 0.534$). There was also no statistically significant difference between genotypes in the percentage of Ki67-positive primary follicles in mice fed the 600 mg/kg ALA diet ($P = 0.236$), but ovaries from mice with this level of ALA supplementation had significantly higher percentages of Ki67-positive primary follicles than *Gclm*$^{+/+}$ mice fed the control diet ($P \leq 0.025$). As expected, essentially all secondary and antral follicles had Ki67-positive granulosa cells regardless of experimental group (data not shown).

Effects of Gclm genotype- and ALA diet on ovarian follicle apoptosis

To determine whether the apparent beneficial effect of 150 mg/kg diet ALA supplementation on follicle numbers in *Gclm*$^{-/-}$ mice could be due to decreased follicle apoptosis, we performed immunostaining for the activated, cleaved form of the so-called executioner caspase, caspase-3 (Figure 5). Percentages of secondary follicles with cleaved caspase-3-positive granulosa cells differed significantly by ALA diet group ($P = 0.028$, effect of diet, two-way ANOVA), but not by genotype or genotype by diet interaction, with the percentages of secondary follicles with cleaved caspase-3 positive granulosa cells increasing with increasing ALA concentration in ovaries of both

Table 2. Effects of ALA supplementation on estrous cycling

Diet	Genotype	Regular cycle, % [a]	Diestrous % time	Proestrous % time	Estrous % time	Cycle length (days if cycling)
Control	*Gclm* $^{-/-}$	100	55 ± 4	20 ± 2	24 ± 3	5 ± 0.4
	Gclm $^{+/+}$	87.5	41 ± 3	24 ± 3	36 ± 3	5 ± 0.2
150 mg/kg ALA	*Gclm* $^{-/-}$	100	54 ± 6	21 ± 4	25 ± 8	5 ± 0.4
	Gclm $^{+/+}$	100	46 ± 2	24 ± 2	36 ± 3	5 ± 0.3
600 mg/kg ALA	*Gclm* $^{-/-}$	87.5	55 ± 5	23 ± 3	21 ± 3	5 ± 0.4
	Gclm $^{+/+}$	62.5	63 ± 6	21 ± 3	16 ± 4	6 ± 1.0

[a]Percent of mice with regular 4 or 5 day estrous cycle.

genotypes (Figure 5B). No statistically significant diet or genotype related differences were observed in the percentages of antral follicles with cleaved caspase-3 positive granulosa cells ($P > 0.05$; Figure 5C). Cleaved caspase-3 immunostaining was not observed in primary and primordial follicles (data not shown).

ALA diets rescued increased DNA damage in ovarian follicles of Gclm$^{-/-}$ mice

Histone H2AX is phosphorylated at sites of double strand DNA breaks; once phosphorylated, it is called γH2AX [50]. We used nuclear immunohistochemical staining for γH2AX as a marker of DNA damage in the ovaries (Figure 6A). There were no statistically significant differences in the percentages of primary follicles with positive granulosa cells among groups (Figure 6C). Primordial follicles very rarely had granulosa cells positive for γH2AX (data not shown). There were statistically significant effects of genotype and genotype by diet interactions in the percentages of secondary follicles with γH2AX-positive granulosa cells ($P = 0.004$, genotype; $P = 0.049$, genotype × treatment, two-way ANOVA; Figure 6D), while the main effect of diet approached statistical significance ($P = 0.063$). Intergroup comparisons showed that the percentage of secondary follicles with γH2AX-positive granulosa cells was increased in *Gclm*$^{-/-}$ mice compared with *Gclm*$^{+/+}$ mice on control diet, but not on the ALA diets. There was a statistically significant genotype by diet interaction on percentages of antral follicles with γH2AX-positive granulosa cells ($P = 0.010$, Figure 6E), while the main effects of genotype and diet were not statistically significant ($P = 0.541$ and $P = 0.408$, respectively). Intergroup comparisons showed that the percentages differed between ovaries from *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ mice fed the control diet, but not for mice fed either ALA diet.

The effects of dietary ALA supplementation and diet by genotype interactions on percentages of primordial follicles with γH2AX-positive oocytes approached significance ($P = 0.054$ and $P = 0.058$, respectively, by two-way ANOVA; effect of group $P = 0.050$ by Kruskal–Wallis test; Figure 6F). Intergroup comparisons supported that the percentages of primordial follicles with γH2AX-positive oocytes differed between *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ ovaries in mice fed the control diet, but not the ALA diets (Figure 6F). The percentages of primary follicles with γH2AX-positive oocytes varied significantly with ALA by genotype interaction ($P = 0.009$) and the effect of ALA alone approached significance ($P = 0.086$). Intergroup comparisons showed that percentages of primary follicles with γH2AX-positive oocytes were higher in ovaries of *Gclm*$^{-/-}$ compared with *Gclm*$^{+/+}$ mice on the control diet ($P = 0.002$; Figure 6G), while the percentages did not differ between *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ ovaries on the 150 or 600 mg/kg ALA diets. There were no statistically significant differences in percentages of secondary or antral follicles with γH2AX-positive oocytes among groups (Figure 6H and I).

Compared with the 150 mg/kg ALA diet, the 600 mg/kg ALA diet caused lesser decreases in the percentages of secondary and antral follicles with γH2AX-positive granulosa cells and primordial and primary follicles with γH2AX-positive oocytes in *Gclm*$^{-/-}$ ovaries, while it tended to increase the percentages in *Gclm*$^{+/+}$ ovaries compared with ovaries of the same genotype on the control diet (Figure 6).

N-acetyl cysteine supplementation increased ovarian GSH concentrations in Gclm$^{+/+}$, but not Gclm$^{-/-}$, mice

Supplementation of 2-month old mice with 80 mM NAC in drinking water for 7 days before euthanasia on metestrus of the estrous cycle, slightly, but statistically significantly, increased ovarian total GSH concentrations in *Gclm*$^{+/+}$ females, but had lesser effect on ovarian GSH concentrations in *Gclm*$^{-/-}$ females ($P < 0.001$, effect of genotype; $P = 0.037$, effect of NAC; $P = 0.124$, genotype by NAC interaction; Figure 7A). In contrast, NAC supplementation did not significantly affect ovarian GSSG concentrations, which were significantly lower in *Gclm*$^{-/-}$ mice compared with *Gclm*$^{+/+}$ mice ($P < 0.001$, effect of genotype; $P = 0.248$, effect of NAC; $P = 0.433$, genotype by NAC interaction; Figure 7B). The redox potential of the GSH:GSSG redox couple was more reduced in Gclm$^{+/+}$ ovaries supplemented with NAC compared with control *Gclm*$^{+/+}$ ovaries, while NAC supplementation decreased ovarian GSH:GSSG redox potential to a lesser extent in *Gclm*$^{-/-}$ mice ($P < 0.001$, effect of genotype; $P = 0.004$, effect of NAC; $P = 0.439$, genotype by NAC interaction; Figure 7C).

ALA supplementation did not affect ovarian GSH concentrations

Supplementation with 150 mg/kg or 600 mg ALA per kg diet did not increase ovarian GSH concentrations in *Gclm*$^{+/+}$ or *Gclm*$^{-/-}$ mice (Figure 7D).

Effects of NAC on water intake and weight gain or ALA supplementation on food intake and weight gain

There were no statistically significant effects of *Gclm* genotype or NAC supplementation from PND 21 to 30 on weight gain during the study, but mice given NAC-supplemented water consumed about 2 mL per day less water than those given control water ($P = 0.04$, effect of NAC; Supplemental Table S1). In contrast, there were no statistically significant effects of genotype or NAC supplementation for 7 days in 2 month-old mice on water consumption or body weight (Supplemental Table S2).

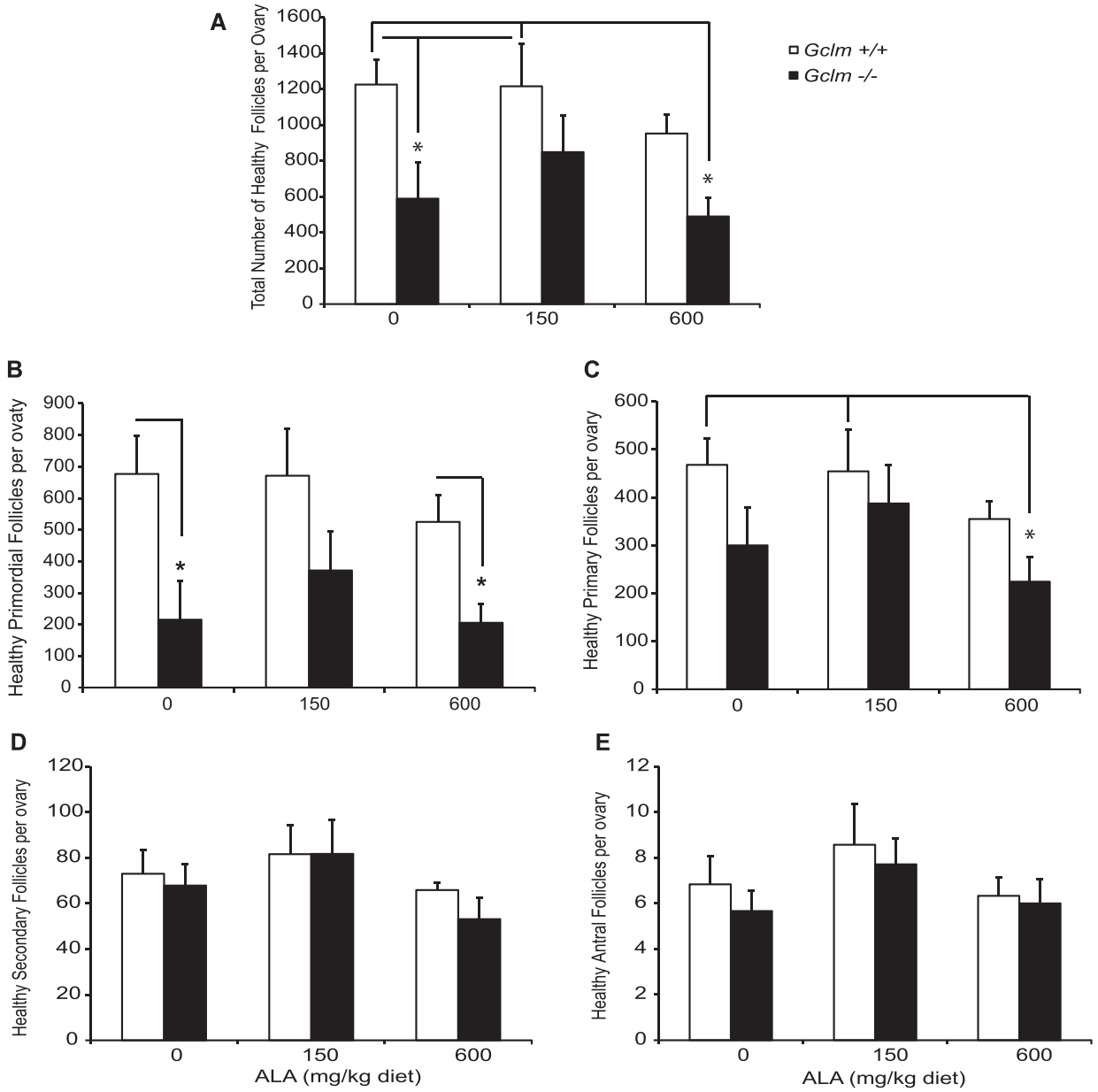

Figure 2. Dietary ALA supplementation partially rescued the decreased ovarian follicle counts in *Gclm*$^{-/-}$ mice. Ovaries were harvested from females in the breeding study described in Figure 1 and were processed for follicle counts as described in Materials and methods. (A) Means ± SEM of the sum of healthy follicles at all stages of development in *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ ovaries at the indicated levels of dietary ALA supplementation ($P = 0.002$, effect of genotype; $P = 0.203$, effect of diet; $P = 0.748$ genotype by diet interaction by two-way ANOVA). (B) Means ± SEM of healthy primordial follicles in *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ ovaries at the indicated levels of dietary ALA supplementation ($P < 0.001$, effect of genotype; $P = 0.378$, effect of diet; $P = 0.748$ genotype by diet interaction by two-way ANOVA). (C) Means ± SEM of healthy primary follicles ($P = 0.037$, effect of genotype; $P = 0.149$, effect of diet; $P = 0.762$ genotype by diet interaction by two-way ANOVA). (D) Means ± SEM of healthy secondary follicles. There were no statistically significant effects of genotype or diet. (E) Means ± SEM of healthy antral follicles. There were no statistically significant effects of genotype or diet. *$P < 0.050$, for the indicated intergroup comparisons. $N = 6$–8/group.

There were no significant differences among groups in the amount of ALA-supplemented diet consumed during the 5 weeks prior to breeding from 3 to 8 weeks of age (Supplemental Table S3). In contrast, there were statistically significant effects of genotype ($P = 0.002$) and genotype by ALA interaction ($P = 0.010$) on food intake between 3 and 10 weeks of age prior to euthanasia for ovarian GSH concentration assay, with the *Gclm*$^{-/-}$ mice consuming less food overall, but tending to consume 0.2 g/day more when on the ALA-supplemented diets, while *Gclm*$^{+/+}$ mice tended to consume 0.1–0.2 g/day less on average on the ALA diets (Supplemental Table S4). There was also a statistically significant effect of genotype and a statistically significant genotype by ALA interaction on weight gain during that period, with *Gclm*$^{-/-}$ mice gaining less weight, but tending to gain 0.4–0.6 g more weight on average on the ALA-supplemented diets, while *Gclm*$^{+/+}$ mice tended to gain 1.3–1.5 g less weight on average on the ALA diets (Supplemental Table S4).

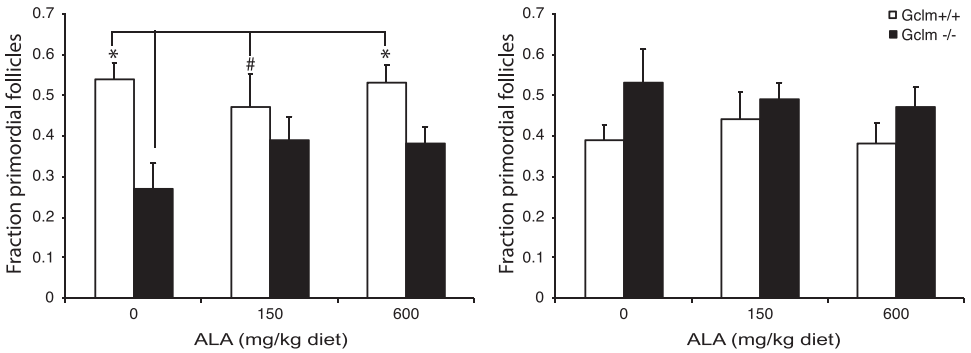

Figure 3. Supplementation with 150 mg/kg diet ALA increased the fraction of primordial follicles in *Gclm*$^{-/-}$ mice compared with *Gclm*$^{-/-}$ mice on the control diet. (A) Fractions ± SEM of primordial follicles of total number of healthy follicles ($P = 0.007$, effect of genotype; $P = 0.686$, effect of diet; $P = 0.279$ genotype by diet interaction by two-way ANOVA). (B) Fractions ± SEM of primary follicles of total number of healthy follicles ($P = 0.056$, effect of genotype; $P = 0.772$, effect of diet; $P = 0.725$ genotype by diet interaction by two-way ANOVA). $^{*}P < 0.050$, $^{\#}P = 0.088$ for the indicated intergroup comparisons. $N = 6$–8/group.

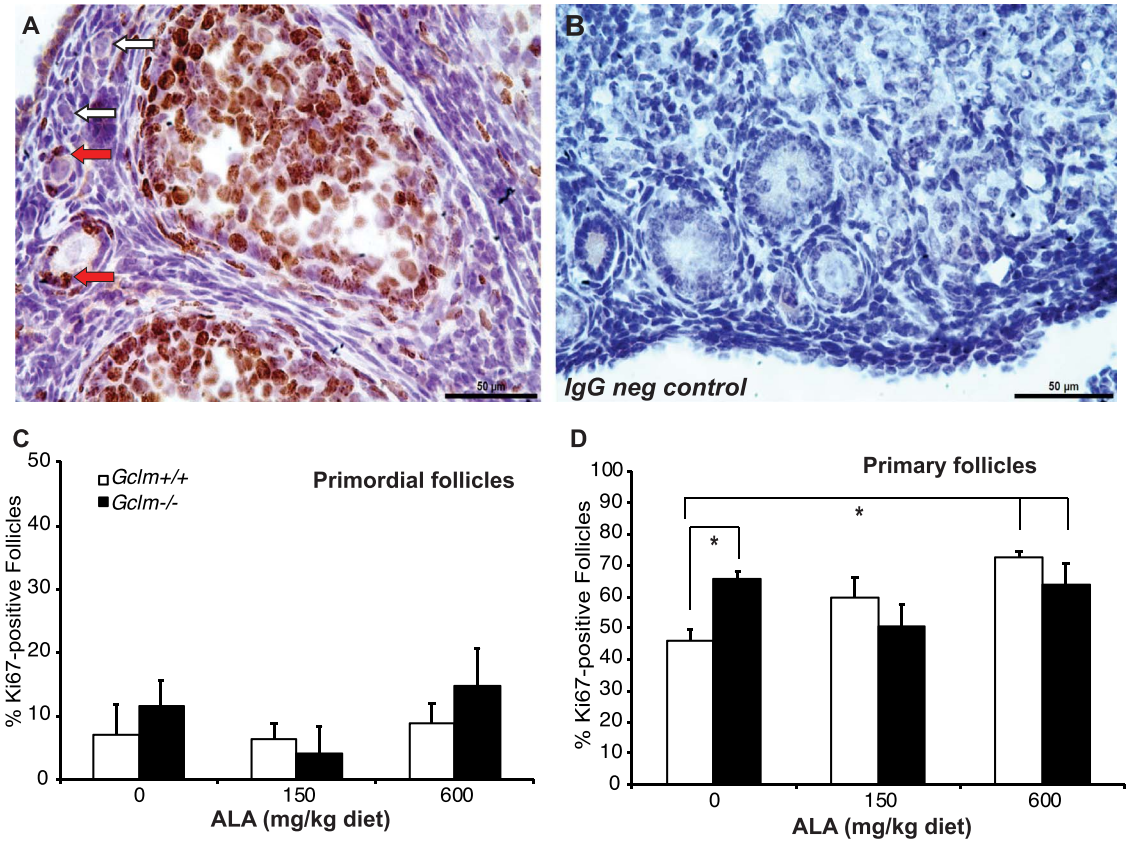

Figure 4. Supplementation with 150 mg/kg diet ALA prevented the increased recruitment of follicles into the growing pool in *Gclm*$^{-/-}$ ovaries. Ovaries harvested from females in the breeding study described in Figure 1 were processed for immunostaining for the mitosis marker Ki67, and follicles with Ki67-positive granulosa cells were scored as described in Materials and methods. (A) Representative Ki67 immunostaining in section from ovary of *Gclm*$^{-/-}$ mouse fed the control diet. Ki67-positive primary follicles with brown stained granulosa cells (red arrows) and Ki67-negative primordial follicles (white arrows) are present. Also seen are portions of larger follicles with numerous Ki67 positive granulosa cells. (B) Technical negative control in which primary antibody was replaced with non-immune serum. The graphs show the means ± SEM percentage of Ki67 positive follicles. (C) There were no statistically significant effects of *Gclm* genotype, dietary ALA concentration, or genotype by diet interaction on percentages of Ki67-positive primordial follicles. (D) The percentages of primary follicles with Ki67-positive granulosa cells varied significantly with dietary ALA concentration and *Gclm* genotype by diet interaction ($P = 0.033$, $P = 0.018$, respectively, by two-way ANOVA). $^{*}P < 0.05$, indicated intergroup comparisons. $N = 4$/group. Scale bars, 50 µm.

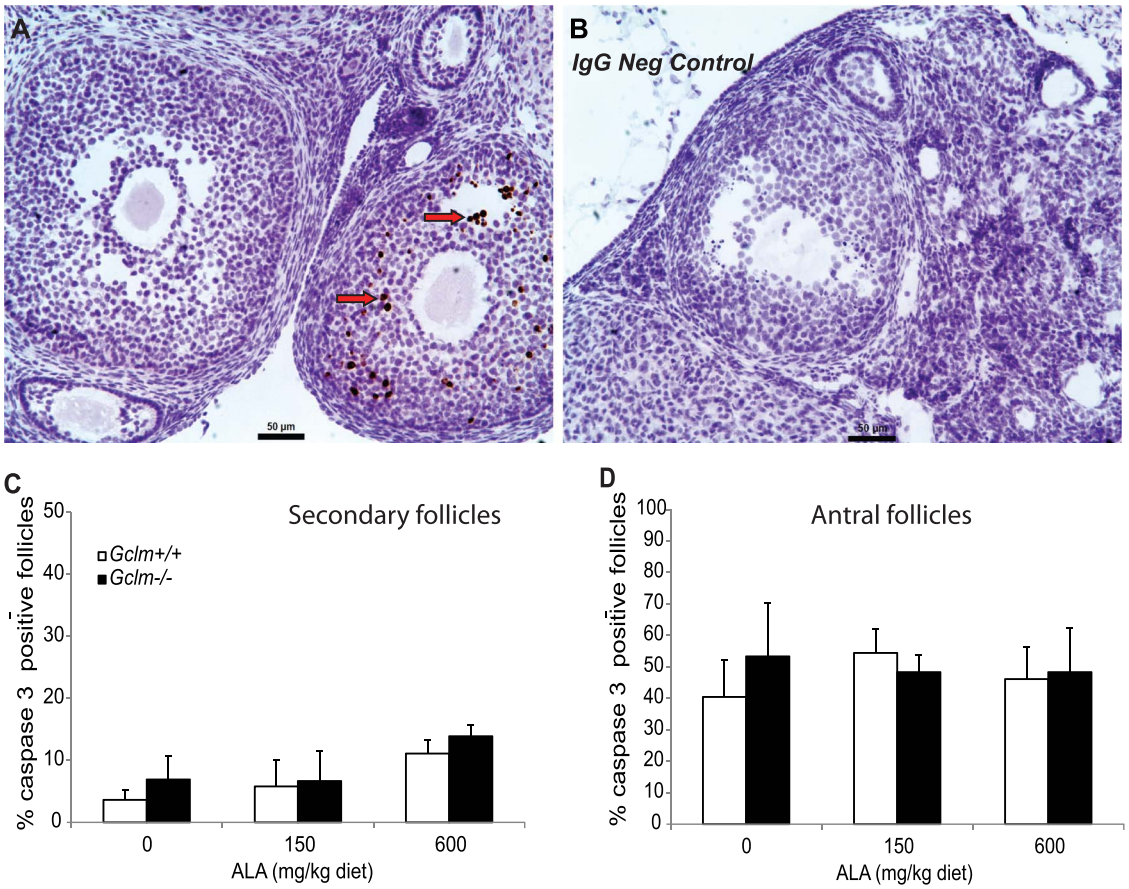

Figure 5. Effects of *Gclm* genotype- and ALA diet on activated caspase 3 in ovarian follicles. Cleaved caspase-3 immunostaining was used to assess apoptosis in ovarian follicles as described in Materials and Methods. (A) Representative cleaved caspase 3 immunostaining in section from ovary of $Gclm^{-/-}$ mouse fed the control diet. Caspase 3-positive follicles have brown stained granulosa cells (red arrows). (B) Technical negative control in which primary antibody was replaced with non-immune serum. The graphs show the means ± SEM percentage of cleaved caspase-3 positive follicles per ovary. (C) Percentages of cleaved caspase-3 positive secondary follicles increased with increasing dietary ALA concentration ($P = 0.028$); pairwise intergroup comparisons were not statistically significant. (D) There were no statistically significant effects of genotype or diet on antral follicle cleaved caspase-3 immunostaining. $N = 5$/group. Scale bars, 50 µm.

The differences in food consumption between the two experiments could be explained by the longer duration of the second experiment or could have arisen by chance.

Discussion

We investigated whether the accelerated age-related decline in ovarian follicles and the preimplantation embryonic mortality resulting in lower offspring production, which we previously reported in GSH-deficient $Gclm^{-/-}$ mice [19, 20], could be rescued by thiol antioxidant supplementation. Our results show that dietary ALA supplementation from weaning until 20 weeks of age with a daily dose similar to that used in humans partially rescued the greater age-related decline in primordial follicle numbers in $Gclm^{-/-}$ mice, while a fourfold higher dose was not beneficial. Further, our results support that the rescue of primordial follicle numbers was due to normalization (to levels comparable with $Gclm^{+/+}$ mice) of recruitment of primordial follicles into the growing pool and normalization of follicular DNA damage in 150 mg ALA/kg diet supplemented $Gclm^{-/-}$ mice. These findings are important because premature depletion of the ovarian follicle reserve is associated with increased risk of osteoporosis, cardiovascular disease, and dementia [51–54], in addition to shortening the fertile lifespan. In contrast, dietary ALA supplementation did not rescue the decreased offspring production, nor did it alter ovarian GSH concentrations, of $Gclm^{-/-}$ female mice. Supplementation with the GSH precursor NAC for 8 days beginning 6 days before initiation of hormonal stimulation for superovulation did not rescue the low rate of second pronucleus formation in zygotes of $Gclm^{-/-}$ females at 0.5 days post-coitum, which we previously found was associated with high embryonic mortality prior to the blastocyst stage [19]. Finally, NAC supplementation slightly, but statistically significantly increased ovarian GSH concentrations in $Gclm^{+/+}$ mice, while having minimal effect on ovarian GSH in $Gclm^{-/-}$ mice.

Gclm−/− females are born with a similar complement of oocytes as wild type littermates, but they experience an accelerated decline in primordial follicles as they age [20]. This appears to be due to increased recruitment of primordial follicles into the growing pool, rather than increased death of primordial follicles [20]. Consistent with our prior findings, we observed significantly decreased primordial and primary follicle numbers, decreased fraction of primordial follicles, and increased percentages of primary follicles with Ki67-positive granulosa cells in Gclm−/− mice fed the control diet, and these genotype-related differences were abolished in Gclm−/− mice

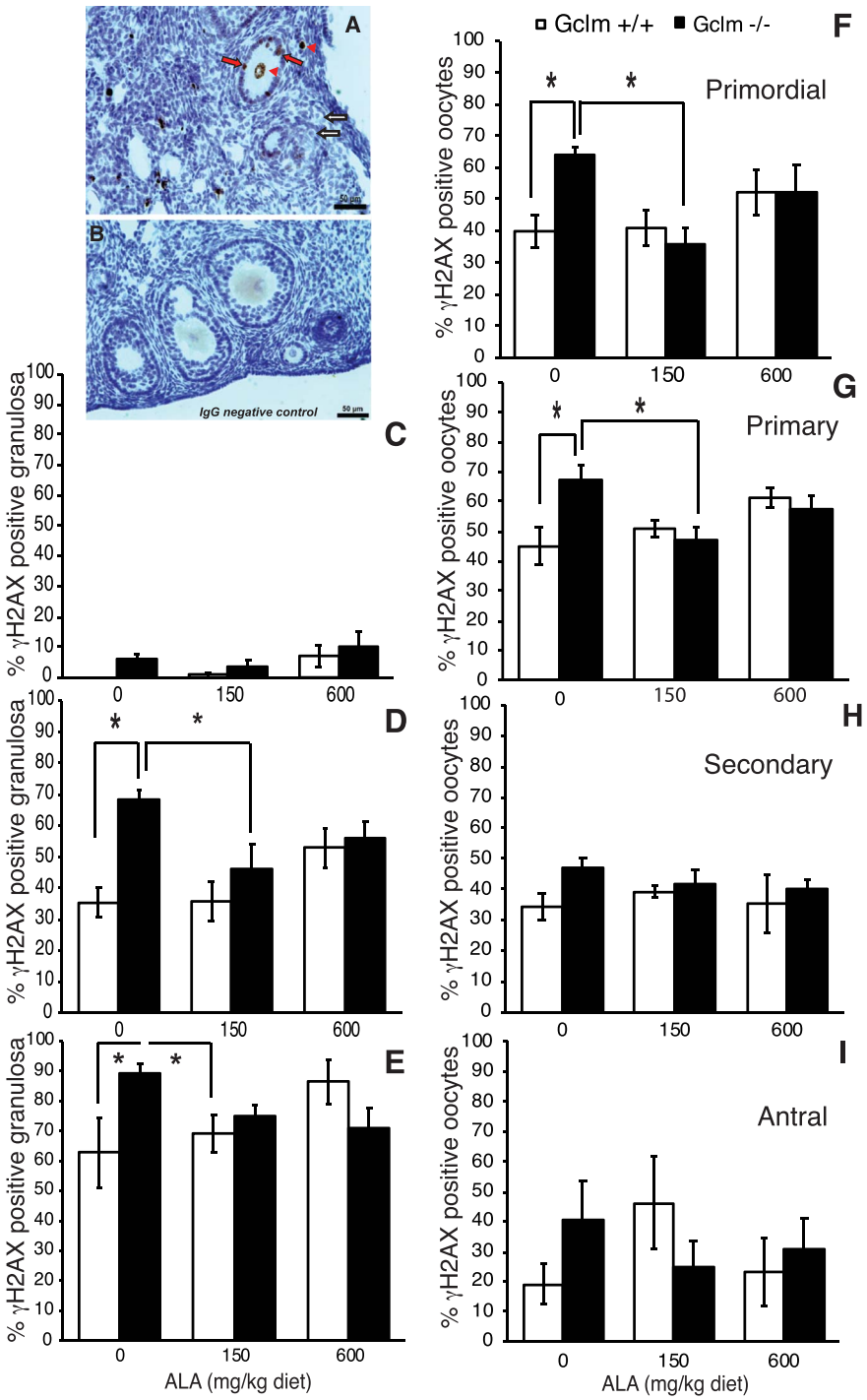

Figure 6. Dietary ALA supplementation rescued increased DNA damage in ovarian follicles of Gclm$^{-/-}$ mice. Nuclear γH2AX immunostaining in oocytes and granulosa cells was scored as described in Materials and methods. Representative image of (A) experimental ovary section from *Gclm*$^{+/+}$ fed the 600 mg ALA/kg diet. Red and white arrows point to γH2AX-positive (dark brown staining) and -negative follicles. Red arrow heads point to γH2AX-positive ooctyes. (B) Technical negative control in which primary antibody was replaced with non-immune serum. The graphs show means ± SEM percentages of follicles of the indicated stage of development with γH2AX-positive oocytes or granulosa cells. (C) There were no statistically significant differences in the percentages of primary follicles with positive granulosa cells among groups. (D) There were statistically significant effects of genotype and genotype by diet interactions in the percentages of secondary follicles with γH2AX-positive granulosa cells ($P = 0.004$, genotype; $P = 0.049$, genotype × diet; $P = 0.063$, diet, two-way ANOVA). (E) There was a statistically significant genotype by diet interaction on percentages of antral follicles with γH2AX-positive granulosa cells ($P = 0.010$), while the main effects of genotype and diet were not statistically significant ($P = 0.541$ and $P = 0.408$, respectively). (F) Effects of dietary ALA supplementation and genotype by diet interaction on primordial follicle oocyte γH2AX immunostaining approached statistical significance ($P = 0.054$ and $P = 0.058$, respectively by two-way ANOVA and $P = 0.050$ by Kruskal–Wallis test). (G) Percentages of primary follicles with γH2AX-positive oocytes varied significantly with ALA by genotype interaction ($P = 0.009$, two-way ANOVA) and the effect of ALA alone approached significance ($P = 0.086$). There were no statistically significant differences in percentages of secondary (H) or antral (I) follicles with γH2AX-positive oocytes among groups. $^*P < 0.05$, indicated intergroup comparison. $N = 5$/group. Scale bars, 50 μm.

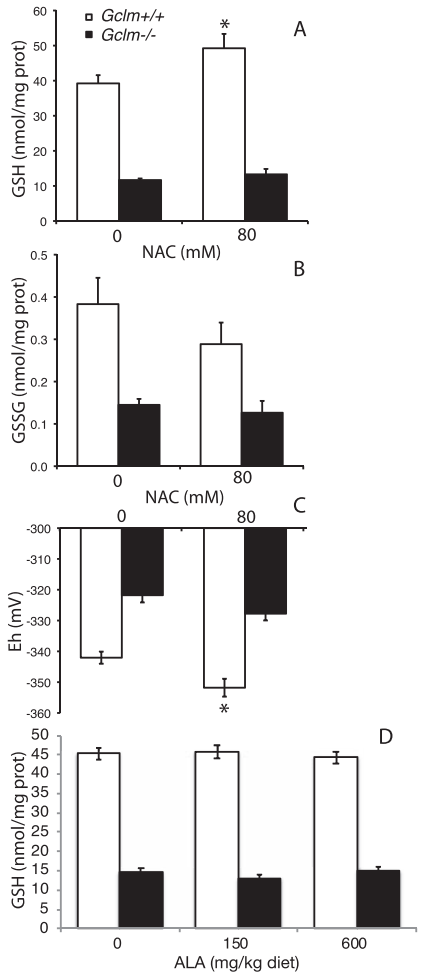

Figure 7. Effects of NAC or ALA supplementation on ovarian concentrations of GSH and GSSG in *Gclm*$^{+/+}$ and *Gclm*$^{-/-}$ females. Total and oxidized GSH were measured and the Eh for GSH/GSSG were calculated as described in *Materials and Methods*. (A–C) *Gclm*$^{-/-}$ mice have significantly decreased ovarian concentrations of total GSH and oxidized GSH (GSSG) and a more oxidized Nernst potential (Eh) of the GSSG/GSH redox couple compared with wild-type littermates regardless of NAC supplementation ($P < 0.001$, effect of genotype for all three endpoints). (A) Mean ± SEM of total ovarian GSH in NAC supplemented and control mice ($P = 0.037$, effect of NAC; $P = 0.124$, genotype by NAC interaction). (B) Mean ± SEM of ovarian GSSG concentrations in NAC supplemented and control mice ($P = 0.248$, effect of NAC; $P = 0.433$, genotype by NAC interaction). (C) Mean ± SEM of Eh for GSH/GSSG redox couple in NAC supplemented and control mice ($P = 0.004$, effect of NAC; $P = 0.439$, genotype by NAC interaction). $^{*}P < 0.02$ versus 0 mM NAC of same genotype. $N = 5$–7/group. (D) Mean ± SEM of total ovarian GSH concentrations in *Gclm*$^{+/+}$ and *Gclm*$^{-/-}$ mice fed diets containing 0, 150, and 600 mg/kg ALA from 3 to 10 weeks of age. Ovarian GSH concentrations were significantly lower in *Gclm*$^{-/-}$ mice compared with *Gclm*$^{+/+}$ mice ($P < 0.001$, effect of genotype), but dietary ALA supplementation did not affect ovarian GSH concentrations ($P = 0.933$, effect of ALA; $P = 0.509$ genotype × ALA interaction). $N = 5$–7/group.

fed the 150 mg/kg ALA diet. Our follicle classification scheme places transitional or activated follicles into the same group as primary follicles. Therefore, the abolition of the *Gclm* genotype-related differences in the fraction of all healthy follicles at the primordial stage and in percentages of Ki67-positive primary follicles in *Gclm*−/− mice fed the 150 mg/kg ALA diet indicates that the 150 mg/kg ALA diet prevents increased recruitment of primordial follicles into the growing pool in *Gclm*−/− mice. These data provide additional support for accelerated recruitment of primordial follicles into the growing pool in *Gclm*−/− ovaries and further support that chronic ovarian oxidative stress may lead to accelerated recruitment of follicles into the growing pool. Consistent with our observation that the 600 mg/kg ALA diet failed to rescue the decreased primordial follicle numbers in *Gclm*−/− mice, the percentages of Ki67-positive primary follicles were higher in ovaries of both *Gclm* genotypes fed that diet compared with ovaries of mice of the same genotype fed control diet or 150 mg/kg ALA diet.

We previously showed that the ovaries of *Gclm*$^{-/-}$ mice are in a state of chronic oxidative stress, with concentrations of GSH decreased to a greater extent than GSSG, resulting in decreased ratio of GSH:GSSG and oxidized Nernst potential of the GSH:GSSG redox couple, as well as increased oxidative lipid and protein damage [20]. The present study further demonstrates that *Gclm*$^{-/-}$ ovaries from mice on the control diet have increased DNA damage, detected by immunostaining for γH2AX, a marker of double strand DNA breaks, in oocytes and granulosa cells of follicles, compared with *Gclm*$^{+/+}$ ovaries on the same diet. Oxidative DNA damage is a known cause of double strand DNA breaks [55]. Consistent with oxidative DNA damage as the cause of the increased γH2AX immunostaining, dietary supplementation with 150 mg ALA/kg diet normalized the increased γH2AX immunostaining in *Gclm*$^{-/-}$ ovaries. In contrast, the 600 mg/kg diet resulted in lesser decreases in γH2AX immunostaining in *Gclm*$^{-/-}$ ovaries and tended to increase γH2AX immunostaining in *Gclm*$^{+/+}$ ovaries compared with the control diet. We previously reported that 150 mg/kg diet ALA supplementation was protective against induction of γH2AX in ovarian follicles by irradiation of mice with heavy iron ions [7], but provided no protection against induction of γH2AX by irradiation with heavy oxygen ions [6]. Similarly, dietary ALA supplementation decreased induction of γH2AX in livers of rats treated with *N*-diethylnitrosamine and thioacetamide in a two-stage model of hepatic carcinogenesis [56]. α-Lipoic acid supplementation by intraperitoneal injection was also protective against DNA damage induced by drinking water administration of potassium dichromate measured using single cell gel electrophoresis (COMET assay) in peripheral blood mononuclear cells and bone marrow cells [57].

In contrast to partial prevention of the accelerated ovarian follicle decline in *Gclm*$^{-/-}$ females by dietary ALA supplementation, we did not observe amelioration by NAC supplementation of the decreased percentage of zygotes with two pronuclei at 0.5 days post-coitum or by ALA of the decreased offspring production in *Gclm*$^{-/-}$ females. Specifically, supplementation with NAC beginning 6 days before eCG injection did not rescue zygote pronuclear formation, and dietary ALA supplementation from 21 days of age did not rescue decreased offspring production in a 12-week continuous breeding assay. We note that NAC treatment did not occur throughout follicular development, beginning only during the later stages of secondary follicle development, which suggested that it may be necessary to initiate antioxidant supplementation beginning at the primordial follicle stage to rescue the increased early embryonic mortality observed during pregnancies of *Gclm*$^{-/-}$ females. Therefore, we conducted a study of long-term supplementation with ALA, which can be administered via the diet. However, ALA supplementation from weaning through euthanasia at 5.5 months of age also failed to rescue this phenotype of *Gclm*$^{-/-}$ mice. Together the current results and our prior work [19] show that embryos from *Gclm*$^{-/-}$ mothers progress to the 2-pronuclei stage at much lower rates by 0.5 dpc and have very low rates of maturation to the blastocyst stage

by 3.5 dpc than embryos from $Gclm^{+/+}$ females. Our prior work further showed that this is due to effects of *Gclm* deletion in the oocyte itself because oocytes from $Gclm^{-/-}$ females fertilized in vitro with $Gclm^{+/+}$ sperm and cultured for 4 days also failed to reach the blastocyst stage [19]. Moreover, work of earlier investigators demonstrated that mature mouse and hamster oocytes contain GSH concentrations of 8–10 mM, higher than GSH concentrations in germinal vesicle or pronuclear stage oocytes [58, 59]. Using biochemical inhibition of GSH synthesis, prior studies demonstrated that GSH is required for sperm nuclear decondensation and subsequent male pronucleus formation in murine, hamster, and bovine eggs [58–60]. This is because GSH reduces the disulfide cross-linking of the sperm protamines, which is required for sperm nucleus reactivation to occur [61]. Although ALA could theoretically take the place of GSH in reducing disulfide bonds in the sperm nucleus, dietary supplementation with ALA does not achieve sufficiently high tissue concentrations of ALA because it is rapidly catabolized [29]. Therefore, we conclude that the disulfide reducing activity of GSH, as opposed to its antioxidant activity, is likely required to rescue the preimplantation mortality observed in $Gclm^{-/-}$ mice.

Based on their average daily food consumption and body weights, mice in our study consumed about 17 mg ALA/kg body weight on the 150 mg/kg ALA diet and 68 mg ALA/kg body weight on the 600 mg/kg diet. Human therapeutic oral doses reported in the literature range from 200 to 1800 mg/day (3–30 mg/kg for a 60 kg woman). Thus, the 600 mg ALA/kg diet dose is more than twofold higher than the highest doses used in humans. There is limited data in the literature concerning potential toxicity of ALA. A 14-year old girl died of multiorgan failure after purposeful ingestion of at least 6000 grams of ALA [62]. A 20-month old boy who accidentally ingested 226 mg/kg ALA developed metabolic acidosis and seizures, but survived [63]. A study in rats showed that intraperitoneal doses of 4.3 or 16.6 µmol/kg ALA both lowered the activity of hepatic biotin-dependent carboxylase enzymes by competing with biotin, to which ALA is structurally similar [64]. Feeding a biotin-deficient diet for 12 weeks has been reported to destroy primary, secondary and antral follicles, disrupt estrous cycling, and increase serum estradiol and progesterone concentrations in mice [65]. In contrast, feeding mice a biotin-deficient diet for 42 days did not disrupt estrous cycling, but increased the incidence of spindle abnormalities in ovulated oocytes (18% in controls versus 59% in deficient), which was not reversed in mice fed a biotin sufficient diet for 20 days after 22 days on a biotin-deficient diet (48%) [66]. While 600 mg/kg diet ALA supplementation would not be expected to mimic severe biotin deficiency, it is plausible that the relatively mild effects on offspring production and lack of beneficial effects on ovarian follicle decline, which we observed in mice fed the 600 mg/kg ALA diet, could be due, at least in part, to competition by ALA with biotin.

Prior in vivo studies have reported that ALA supplementation increases tissue concentrations of GSH by upregulating nuclear translocation of the transcription factor NRF2, a master regulator of expression of multiple antioxidant genes, including *Gclc* and *Gclm*. Intraperitoneal administration of ALA increased concentrations of cysteine and GSH in the brains and livers, but not the hearts, of aged male rats [31, 32]. The ratio of reduced GSH to GSSG was increased in all three tissues [31, 32]. Nuclear levels of the transcription factor NRF2 and its antioxidant response element binding activity were increased in livers of ALA treated rats [32]. Glutamate cysteine ligase catalytic protein levels and GCL enzymatic activity were increased in livers, but not brains, by ALA administration [31, 32]. Intraperitoneal ALA supplementation also increased concentrations of GSH in mitochondria isolated from brains of young and old male rats [38]. Together with our data demonstrating no effects of dietary ALA supplementation on total ovarian GSH concentrations, these prior studies suggest that ALA has varying effects on GSH synthesis in different tissues. It is also possible that ALA supplementation increased the ratio of GSH to GSSG in the ovaries in our study, but we had insufficient sample to measure GSSG. In addition, our observation that the 150 mg/kg ALA supplementation partially reversed the accelerated age-related decline in ovarian follicles without increasing GSH concentrations in the ovaries of $Gclm^{-/-}$ mice is consistent with upregulated expression of other antioxidant defense genes by ALA supplementation due to increased NRF2 signaling [29, 67].

In conclusion, dietary supplementation with the antioxidant ALA beginning at weaning, before ovarian follicle numbers have begun to diverge in $Gclm^{+/+}$ and $Gclm^{-/-}$ mice, partially rescued the accelerated age-related decline in primordial ovarian follicles in $Gclm^{-/-}$ mice. Our results support that both increased follicular oxidative DNA damage and accelerated recruitment of primordial follicles into the growing pool may mediate the accelerated decline in ovarian follicles in $Gclm^{-/-}$ mice. In contrast, supplementation with the antioxidant NAC beginning during the secondary follicle stage or supplementation with ALA throughout postnatal follicle development from the primordial stage onward failed to rescue the abnormal pronucleus formation and resultant decreased offspring production of $Gclm^{-/-}$ females. Together with previous studies [58–61], the latter results support a requirement for the disulfide reducing activity of high GSH concentrations in the oocyte for normal early embryonic development.

Supplementary data

Supplementary data is available at *BIOLRE* online.

Acknowledgments

The authors thank Dr. TJ Kavanagh, University of Washington, for the gift of the original breeding pair of $Gclm^{+/-}$ mice. We thank Johnny Nguyen, Angelica del Rosario, Ryan Ripperdan, and Lizette Nunez Magana for assistance with monitoring of mice during these experiments.

Conflict of interest

The authors have declared that no conflict of interest exists.

References

1. van Noord PAH, Boersma H, Dubas JS, te Velde ER, Dorland M. Age at natural menopause in a population-based screening cohort: the role of menarche, fecundity, and lifestyle factors. *Fertil Steril* 1997; **68**:95–102.
2. Nelson LM. Primary ovarian insufficiency. *N Engl J Med* 2009; **360**:606–614.
3. Sullivan SD, Castrillon DH. Insights into primary ovarian insufficiency through genetically engineered mouse models. *Semin Reprod Med* 2011; **29**:283–298.
4. Rebar RW. Mechanisms of premature menopause. *Endocrinol Metab Clin North Am* 2005; **34**:923–933.
5. Devine PJ, Perreault SD, Luderer U. Roles of reactive oxygen species and antioxidants in ovarian toxicity. *Biol Reprod* 2012; **86**:1–10.

6. Mishra B, Ripperdan R, Ortiz L, Luderer U. Very low doses of heavy oxygen ion radiation induce premature ovarian failure. *Reproduction* 2017; **154**:123–133.
7. Mishra B, Ortiz L, Luderer U. Charged iron particles, typical of space radiation, destroy ovarian follicles. *Hum Reprod* 2016; **31**:1816–1826.
8. Shan XQ, Aw TY, Jones DP. Glutathione-dependent protection against oxidative injury. *Pharmacol Therap* 1990; **47**:61–71.
9. Anderson ME, Luo JL. Glutathione therapy: from prodrugs to genes. *Semin Liver Dis* 1998; **18**:415–424.
10. Murphy MP. Mitochondrial Thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications. *Antioxid Redox Signal* 2012; **16**:476–495.
11. Anathy V, Roberson EC, Guala AS, Godburn KE, Budd RC, Janssen-Heininger YMW. Redox-based regulation of apoptosis: S-glutathionylation as a regulatory mechanism to control cell death. *Antioxid Redox Signal* 2012; **16**:496–505.
12. Kemp M, Go YM, Jones DP. Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free Radic Biol Med* 2008; **44**:921–937.
13. Dalton TP, Chen Y, Schneider SN, Nebert DW, Shertzer HG. Genetically altered mice to evaluate glutathione homeostasis in health and disease. *Free Radic Biol Med* 2004; **37**:1511–1526.
14. Franklin CC, Backos DS, Mohar I, White CC, Forman HJ, Kavanagh TJ. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Mol Aspects Med* 2009; **30**:86–98.
15. Griffith OW, Mulcahy RT. The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. *Adv Enzymol Relat Areas Mol Biol* 1999; **73**:209–267.
16. Dalton TP, Dieter MZ, Yang Y, Shertzer HG, Nebert DW. Knockout of the mouse glutamate cysteine ligase catalytic subunit (Gclc) gene: embryonic lethal when homozygous, and proposed model for moderate glutathione deficiency when heterozygous. *Biochem Biophys Res Commun* 2000; **279**:324–329.
17. Yang Y, Dieter MZ, Chen Y, Shertzer HG, Nebert DW, Dalton TP. Initial characterization of the glutamate cysteine ligase modifier subunit *Gclm (−/−)* knockout mouse: novel model system for severely compromised oxidative stress response. *J Biol Chem* 2002; **277**:49446–49452.
18. McConnachie LA, Mohar I, Hudson FN, Ware CB, Ladiges WC, Fernandez C, Chatterton-Kirchmeier S, White CC, Pierce RH, Kavanagh TJ. Glutamate cysteine ligase modifier subunit deficiency and gender as determinants of acetaminophen-induced hepatotoxicity in mice. *Toxicol Sci* 2007; **99**:628–636.
19. Nakamura BN, Fielder TJ, Hoang YD, Lim J, McConnachie LA, Kavanagh TJ, Luderer U. Lack of maternal glutamate cysteine ligase modifier subunit (*Gclm*) decreases oocyte glutathione concentrations and disrupts preimplantation development in mice. *Endocrinology* 2011; **152**:2806–2815.
20. Lim J, Nakamura BN, Mohar I, Kavanagh TJ, Luderer U. Glutamate cysteine ligase modifier subunit (*Gclm*) null mice have increased ovarian oxidative stress and accelerated age-related ovarian failure. *Endocrinology* 2015; **156**:3329–3343.
21. Anderson ME, Meister A. Glutathione monoesters. *Anal Biochem* 1989; **183**:16–20.
22. Puri RN, Meister A. Transport of glutathione as γ-glutamylcysteinylglycyl ester into liver and kidney. *Proc Natl Acad Sci USA* 1983; **80**:5258–5260.
23. Ramachandran A, Jaeschke H. Mechanisms of acetaminophen hepatotoxicity and their translation to the human pathophysiology. *J Clin Trans Res* 2017; **3**:157–169.
24. Woodhead K, Foex. BET 1: In paracetamol overdose, is oral N-acetylcysteine as effective as intravenous N-acetylcysteine? *Emerg Med J* 2018; **35**:643–645.
25. Lalitha T, Kerem D, Yannai S. Effect of N-acetyl-cysteine, D-penicillamine and buthionine sulfoximine on glutathione levels and CNS oxygen toxicity in rats. *Pharmacol Toxicol* 1990; **66**:56–61.
26. Reliene R, Schiestl RH. Glutathione depletion by buthionine sulfoximine induces DNA deletions in mice. *Carcinogenesis* 2005; **27**:240–244.
27. Zhang F-P, Lau CS, Monks TJ. The cytoprotective effect of N-acetyl-L-cysteine against ROS-induced cytotoxicity is independent of its ability to enhance glutathione synthesis. *Toxicol Sci* 2011; **120**: 87–97.
28. Aldini G, Altomare A, Baron G, Vistoli G, Carini M, Borsani L, Sergio F. N-acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. *Free Radic Res* 2018; **52**:751–762.
29. Petersen Shay K, Moreau RF, Smith EJ, Smith AR, Hagen TM. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. *Biochim Biophys Acta* 2009; **1790**:1149–1160.
30. Hermann R, Mungo J, Cnota PJ, Ziegler D. Enantiomer-selective pharmacokinetics, oral bioavailability, and sex effects of various alpha-lipoic acid dosage forms. *Clin Pharmacol Adv Therap* 2014; **6**: 195–204.
31. Suh JH, Wang H, Liu R-M, Liu J, Hagen TM. *(R)*-α-Lipoic acid reverses the age-related loss in GSH redox status in post-mitotic tissues: evidence for increased cysteine requirement for GSH synthesis. *Arch Biochem Biophys* 2004; **423**:126–135.
32. Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu R-M, Hagen TM. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proc Natl Acad Sci USA* 2004; **101**:3381–3386.
33. Ziegler D, Ametov A, Barinov A, Dyck PJ, Gurieva I, Low PA, Munzel U, Yakhno N, Maus J, Samigullin R. Oral treatment with α-Lipoic acid improves symptomatic diabetic polyneuropathy: the SYDNEY 2 trial. *Diabetes Care* 2006; **29**:2365–2370.
34. Reljanovic M, Reichel G, Rett K, Lobisch M, Schuette K, Möller W, Tritschler H-J, Mehnert G. The ALADIN study group. Treatment of diabetic polyneuropathy with the antioxidant thioctiv acid (α-lipoic acid): A two year multicenter randomized, double-blind placebo-controlled trial (ALADIN II). *Free Radic Res* 1999; **31**:171–179.
35. Bustamante J, Lodge JK, Marcocci L, Tritschler HJ, Packer L, Rihn BH. α-Lipoic acid in liver metabolism and disease. *Free Radic Biol Med* 1998; **24**:1023–1039.
36. Someya S, Xu J, Kondo K, Ding D, Salvi RJ, Yamasoda T, Rabinovitch PS, Weindruch R, Leeuwenburgh C, Tanokura M, Prolla TA. Age-related hearing loss in C57BL/6J mice is mediated by Bak-dependent mitochondrial apoptosis. *Proc Natl Acad Sci USA* 2009; **106**: 19432–19437.
37. Karapinar OS, Pinar S, Özcan O, Özgur T, Dolapçioglu K. Protective effect of alpha-lipoic acid in methotrexate-induced ovarian oxidative injury and decreased ovarian reserve in rats. *Gynecol Endocrinol* 2017; **33**:653–659.
38. Palaniappan AR, Dai A. Mitochondrial ageing and the beneficial role of α-lipoic acid. *Neurochem Res* 2007; **32**:1552–1558.
39. Truong TT, Soh YM, Gardner DK. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod* 2016; **31**:1445–1454.
40. Giordano G, White CC, McConnachie LA, Fernandez C, Kavanagh TJ, Costa LG. Neurotoxicity of domoic acid in cerebellar granule neurons in a genetic model of glutathione deficiency. *Mol Pharmacol* 2006; **70**:2116–2126.
41. NRC. *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Research Council, National Academy of Sciences, National Academies Press; 2011.
42. Tsai-Turton M, Luderer U. Gonadotropin regulation of glutamate cysteine ligase catalytic and modifier subunit expression in the rat ovary is subunit and follicle stage-specific. *Am J Physiol* 2005; **289**:E391–E402.
43. Luderer U, Kavanagh TJ, White CC, Faustman EM. Gonadotropin regulation of glutathione synthesis in the rat ovary. *Reprod Toxicol* 2001; **15**:495–504.
44. Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research (Part B)* 2007; **80**:84–97.
45. Myers M, Britt KL, Wreford NGM, Ebling FJP, Kerr JB. Methods for quantifying follicular numbers within the mouse ovary. *Reproduction* 2004; **127**:569–580.

46. Charleston JS, Hansen KR, Thyer AC, Charleston LB, Gougeon A, Siebert JR, Soules MR, Klein NA. Estimating human ovarian non-growing follicle number: the application of modern stereology techniques to an old problem. *Hum Reprod* 2007; **22**:2103–2110.
47. Pasternack BS, Shore RE. Analysis of dichotomous response data from toxicological experiments involving stable laboratory mouse populations. *Biometrics* 1982; **38**:1057–1067.
48. Li J, Kawamura K, Cheng Y, Liu S, Klein C, Liu S, Duan E-K, Hsueh AJW. Activation of dormant ovarian follicles to generate mature eggs. *Proc Natl Acad Sci USA* 2010; **107**:10280–10284.
49. Scholzen T, Gerdes J. The Ki-67 protein: from the known to the unknown. *J Cell Physiol* 2000; **182**:311–322.
50. Riches LC, Lynch AM, Gooderham NJ. Early events in the mammalian response to DNA double-strand breaks. *Mutagenesis* 2008; **23**: 331–339.
51. Svejme O, Ahlborg HG, Nilsson J-Å, Karlsson MK. Early menopause and risk of osteoporosis, fracture and mortality: A 34-year prospective observational study in 390 women. *BJOG* 2012; **119**:810–816.
52. Silva I, Mor G, Naftolin F. Estrogen and the aging brain. *Maturitas* 2001; **38**:95–100.
53. Shuster LT, Gostout BS, Grossardt BR, Rocca WA. Prophylactic oophorectomy in premenopausal women and long-term health. *Menopause Int* 2008; **14**:111–116.
54. Dubey RK, Imthurn B, Barton M, Jackson EK. Vascular consequences of menopause and hormone therapy: importance of timing of treatment and type of estrogen. *Cardiovasc Res* 2005; **66**:295–306.
55. Cadet J, Ravanat J-L, TavernaPorro M, Menoni H, Angelov D. Oxidatively generated complex DNA damage: tandem and clustered lesions. *Cancer Lett* 2012; **327**:5–15.
56. Kimura M, Fujii Y, Yamamoto R, Yafune A, Hayashi S-M, Suzuki K, Shibutani M. Involvement of multiple cell cycle aberrations in early preneoplastic liver cell lesions by tumor promotion with thioacetamide in a two-stage rat hepatocarcinogenesis model. *Exp Toxicol Pathol* 2013; **65**:979–988.
57. Kumar S, Budhwar R, Nigam A, Priya S. Cytoprotection against Cr(6+)-induced DNA damage by alpha-lipoic acid: implications in reducing occupational cancer risk. *Mutagenesis* 2009; **24**:495–500.
58. Calvin HI, Grosshans K, Blake EJ. Estimation and manipulation of glutathione levels in prepuberal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. *Gamete Res* 1986; **14**:265–275.
59. Perreault SD, Barbee RR, Slott VL. Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. *Dev Biol* 1988; **125**:181–186.
60. Sutovsky P, Schatten G. Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biol Reprod* 1997; **56**:1503–1512.
61. Perreault SD, Wolff RA, Zirkin BR. The role of disulfide bond reduction during mammalian sperm nuclear decondensation in vivo. *Dev Biol* 1984; **101**:160–167.
62. Hadzik B, Grass H, Mayatepek E, Daldrup T, Hoehn T. Fatal non-accidental alpha-lipoic acid intoxication in an adolescent girl. *Klin Padiatr* 2014; **226**:292–294.
63. Karaarslan U, Isguder R, Bag O, Kisla M, Agin H, Unal N. Alpha lipoic acid intoxication, treatment and outcome. *Clin Toxicol* 2013; **51**:522.
64. Zempleni J, Trusty TA, Mock DM. Lipoic acid reduces the activities of biotin-dependent carboxylases in rat liver. *J Nutr* 1997; **127**:1776–1781.
65. Báez-Saldaña A, Camacho-Arroyo I, Espinosa-Aguirre JJ, Neri-Gómez T, Rojas-Ochoa A, Guerra-Araiza C, Larrieta E, Vital P, Diaz G, Chavira R, Fernandez-Mejia C. Biotin deficiency and biotin excess: effects on the female reproductive system. *Steroids* 2009; **74**:863–869.
66. Tsuji A, Nakamura T, Shibata K. Biotin-deficient diet induces chromosome misalignment and spindle defects in mouse oocytes. *Biosci Biotechnol Biochem* 2015; **79**:292–299.
67. Petersen Shay K, Michels AJ, Li W, Kong A-NT, Hagen TM. Cap-independent Nrf2 translation is part of a lipoic acid-stimulated detoxification stress response. *Biochim Biophys Acta* 1823; **2012**:1102–1109.