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Original Contribution

EFFECTS OF CYCLOPHOSPHAMIDE AND BUTHIONINE SULFOXIMINE ON OVARIAN GLUTATHIONE AND APOPTOSIS

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Abstract—Treatment with the anticancer drug cyclophosphamide (CPA) destroys ovarian follicles. The active metabolites of CPA are detoxified by conjugation with glutathione (GSH). We tested the hypotheses that CPA causes apoptosis in ovarian follicles and that suppression of ovarian GSH synthesis before CPA administration enhances CPA-induced apoptosis. Proestrous rats were given two injections, 2 h apart, with (1) saline, then saline; (2) saline, then 50 mg/kg CPA; (3) saline, then 300 mg/kg CPA; or (4) 5 mmol/kg buthionine sulfoximine (BSO) to inhibit glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, and then 50 mg/kg CPA. Statistically significantly increased DNA fragmentation by agarose gel electrophoresis and granulosa cell apoptosis by TUNEL were observed in the CPA-treated ovaries 24 h after the second injection, but BSO did not enhance the effect of 50 mg/kg CPA. We next tested the hypothesis that CPA depresses ovarian GSH concentration and expression of the rate-limiting enzyme in GSH synthesis, GCL. Proestrous rats were injected with 300 or 50 mg/kg CPA or vehicle and were sacrificed 8 or 24 h later. After CPA treatment, ovarian and hepatic GSH levels decreased significantly, and ovarian GCL subunit mRNA levels increased significantly. There were no significant changes in GCL subunit protein levels. Finally, we tested the hypothesis that GSH depletion causes apoptosis in ovarian follicles. Proestrous or estrous rats were injected with 5 mmol/kg BSO or saline at 0700 and 1900 h. There was a significant increase in the percentage of histologically atretic follicles and a nonsignificant increase in the percentage of apoptotic, TUNEL-positive follicles 24 h after onset of BSO treatment. Our results demonstrate that CPA destroys ovarian follicles by inducing granulosa cell apoptosis and that CPA treatment causes a decline in ovarian GSH levels. More pronounced GSH suppression achieved after BSO treatment did not cause a statistically significant increase in follicular apoptosis. Thus, GSH depletion does not seem to be the mechanism by which CPA causes follicular apoptosis. © 2004 Elsevier Inc. All rights reserved.

Keywords—Atresia, Follicle, Ovary, Glutathione, Cyclophosphamide, Apoptosis, Free radicals

INTRODUCTION

Glutathione (GSH) is the most abundant nonprotein thiol in mammalian cells. It plays important roles in maintaining the intracellular environment by assisting with amino acid transport, regulating enzyme activity, maintaining membrane structural integrity, and protecting against exogenous and endogenous toxicants [1]. Although liver and kidney

are the most active tissues in producing and using GSH, ovarian cells also contain moderately high levels of GSH [2,3]. It is likely that ovarian GSH plays an important role in protecting ovarian follicles from damage by exogenous toxicants. Cellular levels of GSH are maintained by two tightly coupled enzymatic processes involving a rate-limiting step, catalyzed by glutamate cysteine ligase (GCL), to form γ -glutamylcysteine, followed by addition of glycine to make glutathione [4,5]. The enzymatically active form of GCL is a heterodimer, composed of a catalytic (GCLc) and a modifier (GCLm) subunit [4,5].

The antineoplastic drug cyclophosphamide (CPA) has been widely used in treating patients with various types

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of cancer and autoimmune diseases for some time. Unfortunately, premature menopause occurs in many women treated with CPA [6,7]. Studies in rodents have shown that CPA causes a dose- and time-dependent depletion of ovarian follicles [8–10]. The histological appearance of degenerating follicles after CPA treatment has been reported to resemble that of follicles undergoing the natural process of degeneration termed atresia, with pyknosis of granulosa cell nuclei and separation of the oocyte from the cumulus cells [10,11]. Atresia is an apoptotic process whereby the majority of mammalian ovarian follicles degenerate before ovulation [12]. Other indicators of ovarian apoptosis, such as oligonucleosomal DNA fragmentation, have not been examined after CPA treatment.

CPA itself is biologically inactive and requires metabolic activation. The two main active metabolites of CPA are phosphoramidate mustard and the α,β -unsaturated aldehyde, acrolein [13]. Both of these metabolites are potent cytotoxic agents, which exert their biological effects through covalently binding to important cellular macromolecules. Both phosphoramidate mustard and acrolein are detoxified by spontaneous or glutathione *S*-transferase-mediated conjugation with GSH [13]. These GSH conjugates are excreted, resulting in a net loss of GSH. Phosphoramidate mustard possesses DNA-alkylating activity and is generally considered to be the therapeutically significant cytotoxic metabolite of cyclophosphamide [13]. Phosphoramidate mustard has also been shown to be the metabolite responsible for follicle depletion in the mouse ovary [14].

CPA treatment has been reported to dose-dependently deplete GSH levels in liver, lung, and bone marrow [15–18]. We have previously demonstrated, using *in situ* hybridization, that *Gclm* mRNA is strongly expressed in the granulosa cells and oocytes of healthy, growing follicles, whereas atretic follicles, which have granulosa cells that stain for DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), lack *Gclm* expression [19]. Loss of *Gclm* expression would deplete GCL holoenzyme, turning off GSH synthesis. Taken together these findings led us to hypothesize that ovarian GSH depletion may induce follicular apoptosis and that this may be the mechanism by which CPA causes destruction of follicles.

In the present study we tested the following hypotheses: (1) CPA treatment increases ovarian follicular apoptosis; (2) suppression of ovarian GSH synthesis before CPA administration enhances CPA-induced apoptosis; (3) CPA treatment depresses ovarian GSH concentrations and suppresses protein or mRNA expression of GCL, the rate-limiting enzyme in GSH synthesis; and (4) GSH depletion increases apoptosis in ovarian follicles.

MATERIALS AND METHODS

Animals

Eight to nine week old Sprague Dawley female rats (CrI:CD(SD)IGS BR) were purchased from Charles River Laboratories. Upon arrival, the animals were housed in an AAALAC-accredited facility, three to a cage, with free access to deionized water and standard laboratory chow, on a 14 h/10 h light/dark cycle. After a 7 day acclimatization period, daily vaginal cytology was performed for at least two 4 day estrous cycles before initiation of the experiments. The experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* [20] and the experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of California at Irvine.

Experimental design

For all experiments, animals were randomly assigned to treatment groups using a random number table. Proestrous adult female rats weighing 212 to 232 g were injected i.p. with (1) 5 mmol/kg buthionine sulfoximine (BSO; Sigma–Aldrich, St. Louis, MO, USA), a specific inhibitor of GCL activity, in 0.9% saline, followed 2 h later by 50 mg/kg CPA (Sigma–Aldrich) in 0.9% saline/10% DMSO (Sigma–Aldrich); (2) saline alone followed 2 h later by 300 mg/kg CPA in saline/DMSO; (3) saline followed 2 h later by 50 mg/kg CPA in saline/DMSO; or (4) saline alone followed 2 h later by saline/DMSO. The animals were sacrificed by decapitation 24 h after the second injection, and ovaries were processed for DNA extraction and agarose gel electrophoresis for detection of DNA fragmentation, TUNEL for *in situ* detection of apoptotic cells, or GSH assay. The two doses of CPA were chosen to cause a moderate and a pronounced degree of antral follicle degeneration, respectively, based on previous studies [10,11].

In the second experiment, rats weighing 192 to 308 g were injected intraperitoneally with CPA dissolved in saline with 10% DMSO at a dose of 50 or 300 mg/kg of body weight or with an equivalent volume of saline/DMSO alone at 0800–0900 h on proestrus, as determined by vaginal cytology. Eight or 24 h after injection, the animals were rapidly killed by decapitation, trunk blood was collected, and ovaries were removed and processed for GSH assay or frozen on dry ice and stored at -70°C until protein or RNA extraction. Blood was allowed to clot at room temperature and was centrifuged, and serum was collected and stored at -20°C until estradiol and progesterone assay.

For the third experiment proestrous or estrous rats weighing 208 to 295 g were injected i.p. with 5 mmol/kg BSO in 0.9% saline or with saline alone at 0700 and

1900 h and sacrificed 24 h after the first injection. Ovaries were removed and processed for DNA extraction and electrophoresis, TUNEL, or GSH assay. Liver fragments were also processed for GSH assay. These two estrous cycle stages were chosen to test the effect of GSH suppression on a day when ovarian GSH levels were high (estrus) and on a day when they were low (proestrus) [3].

In all of the experiments estrous cycle stage was further verified at autopsy by checking for the presence of uterine ballooning, which occurs on proestrus, followed by loss of uterine fluid estrous morning [21]. The uterus was dissected out without puncturing it, weighed, slit open and blotted dry to remove any fluid contained within it, and weighed again. Uteri with 100 mg or more fluid were considered ballooned.

Glutathione assay

Total glutathione (reduced plus oxidized glutathione) was measured by a modification of an enzymatic assay developed by Griffith [22]. Immediately after sacrifice, the ovary was homogenized in 5% sulfosalicylic acid on ice (1:4 w/v) and centrifuged at $15,800 \times g$, and the supernatant was removed and stored at -70°C until assay as described [19]. The interassay coefficient of variation is 13.6% for a rat liver pool.

Estradiol and progesterone assays

A standard curve was prepared in charcoal-stripped, ovariectomized rat serum using a stock solution of 17β -estradiol (Sigma, St. Louis, MO, USA) dissolved in 100% ethanol at a concentration of 100 ng/ml that was serially diluted to concentrations of 10, 20, 50, 150, 500, and 1000 pg/ml. The other reagents were from the Estradiol Double Antibody Radioimmunoassay Kit (Diagnostic Products Corp., Los Angeles, CA, USA), and the assay was performed according to the manufacturer's protocol. The standard curve generated in rat serum was demonstrated to be parallel to serial dilutions of a proestrous rat serum pool (data not shown). The interassay coefficient of variation (CV) was 8.5% and the intra-assay CV was 9.0% for an estrous morning (0900 h) serum pool. The progesterone assays utilized the Progesterone CL Radioimmunoassay Kit (Diagnostic Systems Corp., Webster, TX, USA). The standard curve provided with the kit was demonstrated to be parallel to serial dilutions of a proestrous rat serum pool (data not shown). The interassay CV was 14.2% and the intra-assay CV was 8.4% for a high progesterone serum pool (estrus 0000 h).

DNA extraction and agarose gel electrophoresis

Extraction of DNA from whole ovaries was performed using the Qiagen DNeasy kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's instructions. Ten micrograms of DNA from each ovary

was then loaded onto a 2% agarose gel with 0.5 $\mu\text{g/ml}$ ethidium bromide and electrophoresed in Tris acetate EDTA buffer with 0.5 $\mu\text{g/ml}$ ethidium bromide at 50 V for 3.5 h. After electrophoresis, gels were visualized and photographed under ultraviolet light for detection of oligonucleosomal DNA fragmentation. Photographs were scanned using a Hewlett–Packard Scanner. For statistical analysis the mean density of the low-molecular-weight portion of each lane was measured on the scanned images using NIH Image (version 1.60b7).

TUNEL

Ovaries were prefixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 1 h and then immersed for 3 h in 15% sucrose in PBS at 4°C . The

Fig. 1. CPA treatment increases ovarian apoptosis. Proestrous female rats received sequential i.p. injections 2 h apart: 0.9% saline followed by 0.9% saline/10% DMSO (sal/sal), saline followed by 50 mg/kg CPA in saline/DMSO (sal/50), saline followed by 300 mg/kg CPA (sal/300), or 5 mmol/kg BSO followed by 50 mg/kg CPA (BSO/50). The animals were sacrificed 24 h later on estrous morning. DNA from one ovary of each animal was separated by electrophoresis with ethidium bromide staining and was visualized under ultraviolet light. A representative gel is shown at top. M, 25 bp DNA step ladder. Mean densities ($\pm$ SEM) obtained using NIH Image of the low-molecular-weight portions of each lane from scans of two gels are shown in the graph at bottom. ANOVA revealed a significant effect of treatment ($p = .038$; $n = 4$ per group). *Significantly different from saline control by Fisher's LSD test.

ovary was then embedded in Tissue Tek OCT (Sakura Finetek USA, Torrance, CA, USA) and stored at -70°C until sectioning. Paraformaldehyde-prefixed ovaries were

sectioned at $10\text{ }\mu\text{m}$ onto charged microscope slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA) using a cryostat and stored at -70°C . TUNEL

Fig. 2. CPA treatment dose-dependently increases apoptosis in granulosa cells of preantral and antral follicles. The experimental protocol was as described for Fig. 1, except that ovaries were fixed, embedded, and serially sectioned, and every fourth section was subjected to TUNEL for in situ detection of DNA fragmentation, as detailed under Materials and Methods. Apoptotic, TUNEL-positive cells are stained brown. (A) Control ovary from animal treated with sequential saline injections. Original magnification $\times 66$. (B) Ovary from animal treated with saline followed by 300 mg/kg CPA. Two antral follicles (1, 2) and a secondary follicle (3) are shown. Original magnification $\times 66$. (C) Secondary follicle from animal treated with saline followed by 300 mg/kg CPA. Original magnification $\times 132$. (D) Secondary follicle from animal treated with saline followed by 50 mg/kg CPA. Original magnification $\times 132$. (E) Ovary from animal treated with 5 mmol/kg BSO, followed by 50 mg/kg CPA. Small antral follicle (1) and large antral follicle (2) are shown. Original magnification $\times 66$. (F) Mean $\pm$ SEM percentage of TUNEL-positive secondary follicles (3 or more TUNEL-stained granulosa cells per cross section) or histologically atretic secondary follicles (3 or more pyknotic nuclei per cross section) in each of the four treatment groups. The overall effect of treatment on arcsine-transformed data was statistically significant by ANOVA ($p < .001$). *Statistically different from respective saline control by Fisher's LSD test. (G) Mean $\pm$ SEM percentage of TUNEL-positive or histologically atretic antral follicles in each of the four treatment groups. The overall effect of treatment on arcsine-transformed data was statistically significant by ANOVA ($p < .001$). *Statistically different from respective saline control by Fisher's LSD test. $N = 3$ per group.

was performed as described on every fourth serial section from each ovary [19].

Scoring of follicular atresia using TUNEL and histological criteria

Serial sections of ovaries were examined under light microscopy and follicles were classified as primary (Pederson stages 1–3), secondary/preantral (Pederson stages 4 and 5), or antral (Pederson stages 6–8) [23,24]. The follicle diameter was measured as the average of two perpendicular diameters in the section in which the oocyte nucleus was visible to avoid scoring any follicle more than once. For follicles in late stages of atresia, the diameters were measured in the section in which the oocyte was visible. Follicles were scored as atretic based upon the following histological criteria that were adapted from Hirshfield [25,26]: (1) more than 3 pyknotic granulosa cell nuclei in any one cross section, except for follicles of fewer than 50 cells in cross section, in which case 1 pyknotic nucleus; (2) separation of the oocyte from the surrounding granulosa cells; (3) loss of distinct boundary between granulosa and theca cell layers. Follicles were judged definitely atretic if they met the pyknosis criteria and were judged as advanced atresia if they met at least two of the remaining criteria. Follicles were judged possibly atretic if they met one of the other criteria, but not the first criterion. Finally, follicles were scored in terms of TUNEL staining: (1) no TUNEL-positive granulosa cells, (2) two or fewer strongly TUNEL-positive granulosa cells in a cross section, or (3) three or more strongly TUNEL-positive granulosa cells in a cross section. The investigator scoring the slides was blinded to the treatment.

Western blot analysis

Protein extraction, electrophoresis, Western blotting for GCLc and GCLm, and semiquantitative image analysis were performed as described [3].

Northern blot analysis

RNA extraction, electrophoresis, and Northern blotting for *Gclc*, *Gclm*, and *Gapdh* were performed as described [3]. Visualization was by autoradiography. Semiquantitative analysis of autoradiographs was performed using a Stratagene molecular documentation and image analysis system. For *Gclm* the optical densities of the two bands were summed for each lane. *Gclc* and *Gclm* optical densities were then normalized to *Gapdh* control and relative differences among treatment groups were calculated.

Statistical analysis

Analysis of variance (ANOVA) was used to determine significant differences in GSH concentration,

body and ovarian weights, GCLc and GCLm protein levels, *Gclc* and *Gclm* mRNA levels, mean density of low-molecular-weight DNA, and percentages of apoptotic follicles among the different doses of CPA and BSO. For the latter analyses, the proportions were first subjected to arcsine transformation [27]. Assay number was included as an additional independent variable in the analyses as appropriate. Post hoc comparisons of CPA- or BSO-treated groups with the control groups were made using Fisher's Least Significant Difference test. The Pearson χ^2 test and the Linear-by-Linear Likelihood test were used to test the hypothesis that the presence of uterine ballooning on estrous morning differed by CPA-treatment group. Results were considered statistically significant if $p < .05$.

Fig. 3. Effects of CPA treatment on ovarian and hepatic GSH concentrations. Proestrous female rats received i.p. injections of 0.9% saline/10% DMSO, 50 mg/kg CPA in saline/DMSO, or 300 mg/kg CPA in saline/DMSO. The animals were sacrificed 8 h later on proestrous evening (Pro) or 24 h later on estrous morning (Est). Tissues were subjected to GSH assay as detailed under Materials and Methods. (A) CPA treatment suppressed ovarian GSH concentrations at 24 h ($p = .016$, effect of treatment by two-way ANOVA, *300 mg/kg different from control by Fisher's LSD test; $n = 11-13$ per group), but not at 8 h after injection ($p = .59$; $n = 4-6$ per group). (B) CPA treatment suppressed hepatic GSH concentrations at 8 h ($p = .024$, effect of treatment by one-way ANOVA, *300 mg/kg different from control by Fisher's LSD test; $n = 4-6$ per group), but not at 24 h, after injection ($p = .32$; $n = 5$ or 6 per group).

RESULTS

Effects of CPA and BSO plus CPA on ovarian oligonucleosomal DNA fragmentation and in situ DNA fragmentation (TUNEL)

CPA caused an increase in oligonucleosomal DNA fragmentation in DNA extracts of whole ovaries collected 24 h after treatment (Fig. 1). Significantly increased DNA fragmentation compared to controls was observed in the 300 mg/kg CPA-treated ovaries (Fig. 1). Pretreatment with 5 mmol/kg BSO 2 h before 50 mg/kg CPA injection or treatment with 50 mg/kg CPA did not significantly increase ovarian DNA fragmentation (Fig. 1).

Fig. 4. Effects of CPA on ovarian GCLc and GCLm protein levels. The experimental protocol was as described for Fig. 3. Western analysis using anti GCLc and GCLm antibodies was performed as detailed under Materials and Methods. The means $\pm$ SEM of the GCLc and GCLm optical densities expressed as fold control are depicted in the graphs. Representative blots are shown above each graph. There was no statistically significant effect of CPA treatment on protein levels of either subunit at (A) 8 or (B) 24 h after injection. $N = 6-8$ per group.

Fig. 5. Effects of CPA on ovarian *Gclc* and *Gclm* mRNA levels. The experimental protocol was as described for Fig. 3. Northern analysis using 32 P-labeled cDNA probes for *Gclc*, *Gclm*, and *Gapdh* was performed as detailed under Materials and Methods. *Gclc* and *Gclm* optical densities were normalized to *Gapdh*. The *Gcl* subunit to *Gapdh* ratios were then divided by the mean ratio for the control lanes on the same blot. The means $\pm$ SEM of the *Gclc* and *Gclm* to *Gapdh* ratios expressed as fold control are depicted in the graphs. Representative blots are shown above the graphs. (A) There was a statistically significant effect of CPA treatment on *Gclc* and *Gclm* mRNA levels at 8 h after injection ($p = .029$, $p = .013$, respectively, effect of treatment by ANOVA; *significantly different from respective control and 50 mg/kg group by Fisher's LSD test; $n = 8$ or 9 per group). (B) There was no statistically significant effect of CPA treatment on mRNA levels of either subunit 24 h after injection. $N = 10-12$ per group.

Table 1. The Effects of BSO Treatment on GSH Concentrations in Ovary and Liver

	GSH concentration (nmol/mg tissue, mean $\pm$ SD)			
	Metestrus		Estrus	
	Saline	BSO	Saline	BSO
Ovary ^a	3.4 $\pm$ 0.8 (11)	1.6 $\pm$ 0.3 (8)	3.6 $\pm$ 0.6 (10)	1.4 $\pm$ 0.6 (11)
Liver ^a	7.3 $\pm$ 0.8 (4)	4.4 $\pm$ 0.7 (3)	7.7 $\pm$ 1.3 (4)	3.9 $\pm$ 1.5 (5)

Cycling adult rats were injected i.p. with 5 mmol/kg BSO or saline vehicle twice, 12 h apart, on proestrus or estrus and sacrificed 12 h after the second injection. Ovary and liver were immediately homogenized for total GSH assay using the enzymatic recycling method.

^a $p < .001$, effect of treatment by ANOVA.

TUNEL demonstrated that CPA treatment dose-dependently increased granulosa cell apoptosis in preantral follicles ($p < .001$, effect of treatment) and antral follicles ($p < .001$, effect of treatment) (Fig. 2), but not in primary or primordial follicles (data not shown). Post hoc testing showed that both doses of CPA significantly increased the proportions of TUNEL-positive preantral and antral follicles compared to controls (Figs. 2F and 2G). The percentage of follicles with apoptotic granulosa cells in the 50 mg/kg CPA-treated animals was not enhanced by pretreatment with BSO to depress GSH levels. In addition to being more numerous, apoptotic follicles in the 300 mg/kg CPA group contained more TUNEL-positive granulosa cells, granulosa cells with pyknotic nuclei, apoptotic bodies, and cellular debris than apoptotic follicles in the other treatment groups (Figs. 2A–2E). When the data were analyzed using histological criteria for atresia rather than TUNEL criteria, the results were essentially the same (Figs. 2F and 2G).

Effects of CPA on total ovarian and hepatic GSH levels

CPA treatment suppressed ovarian GSH levels (Fig. 3A) at 24 h after injection ($p = .016$, effect of treatment by two-way ANOVA). In contrast, there was no significant effect of CPA treatment on ovarian GSH concentrations at 8 h after CPA injection ($p = .59$). Ovarian GSH levels were higher on estrous morning than on proestrous morning, as we have previously reported [3]. Hepatic GSH concentrations (Fig. 3B) were significantly suppressed at 8 h after CPA, but not at 24 h ($p = .02$ at 8 h and $p = .32$ at 24 h, effect of treatment by one-way ANOVA). CPA treatment has previously been reported to suppress hepatic GSH concentrations in male rats [17] and mice [15].

Effects of CPA on ovarian GCLc and GCLm protein and mRNA levels

There was no significant effect of CPA treatment on either GCLc or GCLm protein levels at 24 h ($p = .90$, $p = .96$ for GCLc and GCLm, respectively, by ANOVA) or at 8 h ($p = .13$, $p = .14$ for GCLc and GCLm, respectively) (Fig. 4). Similarly, there was no significant effect of CPA treatment on *Gcl* subunit mRNA levels normalized to *Gapdh* at 24 h ($p = .64$, $p = .47$, effect of treatment on *Gclc* and *Gclm*, respectively, by ANOVA) (Fig. 5B). However, there were significant increases in *Gclc* and *Gclm* mRNA levels at 8 h after 300 mg/kg CPA ($p = .029$, $p = .013$, effect of treatment on *Gclc* and *Gclm*, respectively, by ANOVA) (Fig. 5A).

Effects of GSH depletion on ovarian apoptosis

The dose of BSO used (5 mmol/kg) significantly suppressed ovarian and hepatic GSH concentrations by more than 50% compared to control tissues at 12 h after the

Fig. 6. Depletion of GSH by BSO treatment did not increase ovarian oligonucleosomal DNA fragmentation. Adult rats were injected with 5 mmol/kg BSO or saline vehicle at 0700 and 1900 h on the proestrous or estrous stage of the estrous cycle and were sacrificed at 0700 h the following day. DNA was extracted from the ovaries, separated by electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. (A) DNA from ovaries of animals treated with saline or BSO on proestrus and sacrificed on estrus. (B) DNA from ovaries of animals treated with saline or BSO on estrus and sacrificed on metestrus. Lane 1, 25 bp DNA stepladder. There was no statistically significant effect of BSO treatment on DNA fragmentation at either estrous cycle stage (data not shown).

second dose in both metestrous and estrous rats (Table 1). Maximal suppression of GSH synthesis occurs within 2 to 5 h after BSO administration in vivo [28]. Thus, the dosage used maintained ovarian GSH levels at less than half of normal levels over the 24 h period before sacrifice.

There was no significant increase in DNA fragmentation in total DNA extracted from whole ovaries of animals treated with BSO on proestrus or estrus (Fig. 6) compared to saline-treated animals. Because an increase in apoptosis in a subset of follicles might not be detectable in whole ovary homogenates, DNA fragmentation after BSO treatment was also assessed in individual follicles.

Serially sectioned ovaries were evaluated for apoptosis by TUNEL and for histological evidence of atresia. There was rarely evidence of apoptosis or atresia among primary follicles after either saline or BSO treatment; therefore, these follicles were excluded from the subsequent analyses. An increase in atretic antral follicles was observed in the BSO-treated ovaries on both estrous cycle stages, but an increase in atretic secondary follicles was observed only after BSO treatment on metestrus (Fig. 7). The increase in atretic antral follicles by histological criteria after BSO treatment was statistically significant ($p = .008$, effect of treatment; Fig. 7A). The increase in antral follicles with apoptotic granulosa cells by TUNEL was not statistically significant ($p = .108$; Fig. 7B). On both cycle stages, a much higher proportion of the antral follicles was atretic than of the secondary follicles (Figs. 7A and 7B). There was also a significant effect of estrous cycle stage on atresia, with metestrous ovaries having higher rates of antral follicle atresia than estrous ovaries (Fig. 7A).

Systemic effects of CPA and BSO

In the second experiment there was a statistically significant, dose-dependent decrease in body weight in the CPA-treated animals at 24 h after injection ($p = .001$, effect of treatment), with the 300 mg/kg group losing 21.9 ± 3.1 g and the 50 mg/kg group losing 8.9 ± 3.1 g. This trend was already evident at 8 h after injection, but was not statistically significant ($p = .076$). Interestingly, the control animals in this experiment also experienced a small amount of weight loss, which was greater at 8 h (6.5 ± 2.8 g) than at 24 h (3.7 ± 3.0 g), indicating recovery. This seemed to be due to the 10% DMSO component of the vehicle, as the controls in the third experiment, which received saline alone, did not lose weight. There was also a statistically significant weight loss of 7.5 ± 1.4 g after BSO treatment in the third experiment ($p < .001$ by t test). There was no statistically significant effect of CPA or BSO treatment on ovarian weight at 8 or 24 h (data not shown).

Eight of 19 animals dosed with 300 mg/kg CPA had hemorrhagic cystitis at autopsy 24 h after injection. No

Fig. 7. Effects of BSO treatment on atresia in antral and secondary follicles. The experimental protocol was described in the legend for Fig. 6, except that ovaries were serially sectioned and were subjected to TUNEL detection of apoptotic cells and histological evaluation for atresia as detailed under Materials and Methods. The graphs summarize data from four to five animals per treatment group. For ease of interpretation, the data are shown as mean percentage $\pm$ SEM; however, statistical analyses were performed on arcsine transformations. (A) The percentages of atretic antral or secondary (preantral) follicles as judged by histological criteria. The effect of BSO treatment on antral follicles was statistically significant ($*p = .008$ by two-way ANOVA). The effect of estrous cycle stage was significant for antral follicles, but not secondary follicles ($p = .016$, $p = .078$, respectively). (B) The percentages of atretic antral or secondary follicles as judged by TUNEL criteria. The effect of treatment was not significant for antral follicles or for secondary follicles ($p = .108$, $p = .552$, respectively, by two-way ANOVA). The effect of estrous cycle stage was statistically significant for secondary follicles, but not for antral follicles ($p = .026$, $p = .398$, respectively). est/met, animals treated with saline or BSO on estrus and sacrificed on metestrus; pro/est, animals treated on proestrus and sacrificed on estrus.

animals in the 50 mg/kg dose group or at the 8 h time point had visible evidence of hemorrhagic cystitis. This is a known side effect of CPA treatment that is also observed in humans [29].

Effects of CPA on uterine ballooning and serum estradiol and progesterone concentrations

Animals treated with 300 mg/kg CPA on proestrus and sacrificed on estrus had a statistically significantly increased rate of persistence of uterine ballooning on

estrous morning ($p < .001$, linear-by-linear likelihood association). Loss of uterine ballooning occurred by 0900 h on estrus in 80% of saline-treated controls, 76% of the 50 mg/kg CPA-treated animals, and only 22% of the 300 mg/kg CPA-treated animals.

Serum estradiol levels were not significantly altered 8 or 24 h after CPA treatment on proestrous morning. Serum estradiol levels were 311.5 ± 64.6 , 202.9 ± 32.4 , and 229.0 ± 40.5 pg/ml at 8 h and 16.6 ± 1.7 , 17.1 ± 2.8 , and 17.8 ± 3.1 pg/ml at 24 h in the control, 50 mg/kg treated, and 300 mg/kg treated animals, respectively.

Similarly, serum progesterone levels were not affected 8 h after CPA treatment. Serum progesterone levels were 61.5 ± 4.3 , 58.5 ± 4.8 , and 62.3 ± 4.0 ng/ml at 8 h in the control, 50 mg/kg treated, and 300 mg/kg treated animals, respectively. However, in contrast to estradiol, serum progesterone levels were significantly elevated ($p = 0.022$, effect of treatment by ANOVA) in the 300 mg/kg group relative to controls and to the 50 mg/kg group at 24 h after CPA treatment. Progesterone concentrations at 24 h were 28.8 ± 3.7 , 33.0 ± 3.6 , and 43.4 ± 3.2 ng/ml in the control, 50 mg/kg treated, and 300 mg/kg treated animals, respectively.

DISCUSSION

We have demonstrated that CPA treatment on proestrous dose-dependently increased apoptosis in granulosa cells of secondary and antral follicles. Further, CPA treatment reduced ovarian GSH levels, without altering protein levels of either subunit of GCL, its rate-limiting enzyme. In contrast to the absence of an effect on GCL subunit protein levels, CPA treatment increased ovarian GCL subunit mRNA levels. GSH suppression alone, after treatment with an inhibitor of GCL, also modestly increased ovarian follicular atresia, but did not have a statistically significant effect on granulosa cell apoptosis.

CPA seems to target follicles in different maturational stages depending on the species studied. Primordial and primary follicles are dramatically depleted in adult [9] and juvenile [8] mice after a single dose of CPA. Antral follicle numbers are also decreased in mice after CPA treatment [9]. In adult rats, growing follicles seem to be the primary targets, and primordial/primary follicles are not affected [10,11]. Jarrell and co-workers reported that antral follicles were depleted 14 days after CPA treatment on postnatal day 30 [11]. Davis and Heindel reported degeneration of all follicles with diameters between 150 and 390 μm at 24 h after a single dose of CPA on proestrus in rats [10]; this size class includes both preantral and small antral follicles. Within the category of antral follicles, the small antral follicles (≤ 390 μm diameter) have also been reported to be the most affected by CPA treatment in mice [9,10]. The results of the

present experiments using TUNEL to identify apoptotic follicles are in agreement with these previous findings, in that increased apoptosis was observed in preantral and antral follicles, but not in primary or primordial follicles, at 24 h after CPA injection. Within the preantral follicles, the effect of CPA was more pronounced in those with diameters greater than 150 μm in the present study (data not shown). It has been hypothesized that large preantral and small antral follicles are most sensitive to CPA because granulosa cells of these follicles have the highest mitotic index [9,10,30]. A study by Ataya and co-workers provides further evidence that multiplying granulosa cells are the primary targets of CPA in the adult rat ovary. This group demonstrated that CPA administration increased granulosa cell DNA cross-linking, followed by granulosa cell death and decreased serum estradiol levels, an indicator of granulosa cell function [31]. DNA damage is a well-known stimulus of entry into the mitochondrial apoptotic pathway [32], and this may be the stimulus by which CPA initiates apoptosis in granulosa cells.

CPA treatment induces apoptosis in other normal tissues, including hair follicles [33], bone marrow [34], testis [35], and thymus [36], as well as in tumor cells [37]. The teratogenic effects of CPA are also thought to be mediated via induction of apoptosis in sensitive tissues, whereas tissues that are not susceptible to CPA-induced teratogenesis are also resistant to CPA-induced apoptosis [38–41]. Induction of apoptosis by CPA in developing mouse embryos seems to occur via activation of the mitochondrial or damage-induced apoptotic pathway, as evidenced by cytochrome *c* translocation from the mitochondria to the cytoplasm and activation of caspases 9 and 3 [39,40]. Similarly, CPA-induced apoptosis in 9L gliosarcoma cells also seems to occur via the mitochondrial pathway [37]. Taken together, our findings and previous studies suggest the hypothesis that CPA treatment causes follicular degeneration by inducing granulosa cell apoptosis via the damage-induced or mitochondrial apoptotic pathway. This hypothesis remains to be proven.

In the present study, GSH suppression using BSO treatment resulted in increased follicular atresia by histological criteria, but the increase in granulosa cell apoptosis as indicated by TUNEL was not statistically significant. The increase in atresia was more pronounced after treatment with BSO on estrus than after treatment on proestrus. This may be due to estrous cycle-related fluctuations in ovarian GSH. Total ovarian GSH concentrations are significantly higher on estrus and metestrus than on diestrus and proestrus [3]. Therefore, treatment with BSO beginning on estrous morning and continuing through metestrous morning suppresses ovarian GSH levels during the two cycle days when they are normally

highest. In contrast, treatment with BSO on proestrus suppresses ovarian GSH levels during a stage of the cycle when they are low and prevents the increase that normally occurs between proestrus and estrus. These results suggest that a physiological role of increased ovarian GSH levels on estrus and metestrus may be to protect maturing antral follicles that will ovulate at the next estrus from proapoptotic stimuli such as reactive oxygen species.

The extent of ovarian GSH suppression brought about by 300 mg/kg CPA in the present study was about a 24% decrease, compared to a greater than 50% decrease achieved with BSO. In contrast, the increase in apoptosis observed after CPA was much greater than the nonsignificant increase in apoptosis observed after BSO. Additionally, 50 mg/kg CPA, a dose that did not suppress ovarian GSH levels, caused a statistically significant increase in follicular apoptosis. Thus, it is unlikely that the increased follicular apoptosis and atresia after CPA are mediated by the CPA-induced reduction of ovarian GSH levels.

GSH depletion causes apoptosis *in vitro* in immortalized cholangiocytes, glioma cells, and a neuronal cell line [42–44], but not in a pancreatic tumor cell line or in leukemia cell lines [45,46]. In the cell lines in which GSH depletion alone does not cause apoptosis, GSH depletion sensitizes the cells to induction of apoptosis by other stimuli, such as melphalan [45] and ultraviolet light [46]. *In vivo*, prolonged GSH depletion with a sulfur amino acid-deficient diet or repeated administration of phorone, but not acute GSH depletion with a single dose of phorone, has been reported to sensitize hepatocytes to Fas-mediated apoptosis [47]. We observed no enhanced effect of a lower dose of CPA on follicular apoptosis after acute GSH depletion with BSO in the present study. However, we did not investigate the effects of prolonged GSH depletion on the sensitivity of ovarian follicles to CPA treatment.

CPA treatment on proestrus blocked the loss of uterine fluid that normally occurs on estrous morning. This loss of fluid is triggered by the increase in progesterone that occurs during the night of proestrus and early morning of estrus [21]. Progesterone levels on estrous morning, 24 h after treatment, were significantly elevated in the high CPA dose group in the present study. This result suggests that CPA treatment delays the rise (and subsequent fall) in progesterone levels. The stimulus for the rise in progesterone secretion late on proestrus is believed to be the preovulatory LH surge [48]. Although our experiments were not designed to assess the effects of CPA treatment on the preovulatory surge or on ovulation, we observed a larger number of unovulated preovulatory follicles on estrous morning, as well as an absence of oocyte–cumulus complexes in the oviducts, in the 300 mg/kg

animals compared to the controls (data not shown). Thus, it is possible that the preovulatory surge and subsequent ovulation were delayed by CPA treatment. Our observation of a significant effect of CPA treatment on progesterone levels at 24 h contrasts with the lack of an effect of a similar CPA treatment regimen in a study by Davis and Heindel [10]. This discrepancy may be due to the lower dose used in that study (250 mg/kg).

CPA treatment resulted in modestly increased *Gclc* and *Gclm* mRNA levels, but not protein levels, in the ovary. Similarly, other electrophilic toxicants that are conjugated by GSH have been shown to induce GCL subunit mRNA expression. β -Naphthoflavone induced mRNA expression of both *Gclc* and *Gclm* in cultured HepG2 cells [49]. Diethyl maleate induced mRNA expression of both subunits in cultured hepatocytes [50]. The antineoplastic agents cisplatin and melphalan also induced GCL mRNA expression in cultured cells [51,52]. None of these studies investigated the effects of these agents on GCL subunit protein levels. The increase in GCL mRNA levels in the absence of a concomitant increase in GCL subunit protein in the ovary after CPA treatment is of uncertain physiological significance. To our knowledge no studies that investigated the effects of cyclophosphamide treatment on GCL expression in any other tissue or cell line have been published.

In summary, our results demonstrate that CPA destroys ovarian follicles by inducing apoptosis in granulosa cells of secondary and antral follicles. GSH suppression using BSO alone caused a nonsignificant increase in granulosa cell apoptosis and a statistically significant increase in follicular atresia. Although both BSO and CPA treatments cause declines in ovarian GSH levels, the suppression of GSH after BSO treatment is at least 2-fold greater than that after CPA treatment. Because BSO causes a greater suppression of ovarian GSH levels, but a much lesser increase in follicular apoptosis, than CPA, the data do not support the hypothesis that CPA causes follicular apoptosis via GSH depletion.

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REFERENCES

- [1] Deneke, S. M.; Fanburg, B. L. Regulation of cellular glutathione. *Am. J. Physiol.* **257**:L163–L173; 1989.
- [2] Clague, N.; Sevcik, M.; Stuart, G.; Brännström, M.; Janson, P. O.; Jarrell, J. F. The effect of estrous cycle and buthionine sulfoximine on glutathione release from the *in vitro* perfused rat ovary. *Reprod. Toxicol.* **6**:533–539; 1992.

- [3] Luderer, U.; Kavanagh, T. J.; White, C. C.; Faustman, E. M. Gonadotropin regulation of glutathione synthesis in the rat ovary. *Reprod. Toxicol.* **15**:495–504; 2001.
- [4] Griffith, O. W.; Mulcahy, R. T. The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **73**:209–267; 1999.
- [5] Soltaninassab, S. R.; Sekhar, K. R.; Meredith, M. J.; Freeman, M. L. Multi-faceted regulation of γ -glutamylcysteine synthetase. *J. Cell. Physiol.* **182**:163–170; 2000.
- [6] Kumar, R.; Biggart, J. D.; McEvoy, J.; McGeown, M. G. Cyclophosphamide and reproductive function. *Lancet* **June 3**: 1212–1214; 1972.
- [7] Warne, G. L.; Fairley, K. F.; Hobbs, J. B.; Martin, F. I. R. Cyclophosphamide-induced ovarian failure. *N. Engl. J. Med.* **289**: 1159–1162; 1973.
- [8] Shiromizu, K.; Thorgeirsson, S. S.; Mattison, D. R. Effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley Rats, C57BL/6N and DBA/2N mice. *Pediatr. Pharmacol.* **4**:213–221; 1984.
- [9] Plowchalk, D. R.; Mattison, D. R. Reproductive toxicity of cyclophosphamide in the C57BL/6N mouse. 1. Effects on ovarian structure and function. *Reprod. Toxicol.* **6**:411–421; 1992.
- [10] Davis, B. J.; Heindel, J. J. Ovarian toxicants: multiple mechanisms of action. In: Korach, K. S., ed. *Reproductive and developmental toxicology*. New York: Dekker; 1998:373–395.
- [11] Jarrell, J.; Young Lai, E. V.; Barr, R.; McMahon, A.; Belbeck, L.; O'Connell, G. Ovarian toxicity of cyclophosphamide alone and in combination with ovarian irradiation in the rat. *Cancer Res.* **47**:2340–2343; 1987.
- [12] Tilly, J. L.; Robles, R. Apoptosis and its impact in clinical reproductive medicine. In: Fauser, B. C. J. M., et al., eds. *Molecular biology in reproductive medicine*. New York: Parthenon; 1999: 79–101.
- [13] Gamcsik, M. P.; Dolan, M. E.; Andersson, B. S.; Murray, D. Mechanisms of resistance to the toxicity of cyclophosphamide. *Curr. Pharm. Des.* **5**:587–605; 1999.
- [14] Plowchalk, D. R.; Mattison, D. R. Phosphoramidate mustard is responsible for the ovarian toxicity of cyclophosphamide. *Toxicol. Appl. Pharmacol.* **107**:472–481; 1991.
- [15] Adams, D. J.; Carmichael, J.; Wolf, C. R. Altered mouse bone marrow glutathione and glutathione transferase levels in response to cytotoxins. *Cancer Res.* **45**:1669–1673; 1985.
- [16] Patel, J. M.; Block, E. R. Cyclophosphamide-induced depression of antioxidant defense mechanisms of the lung. *Exp. Lung Res.* **8**:153–165; 1985.
- [17] Berrigan, M. J.; Struck, R. F.; Gurtoo, H. L. Lipid peroxidation induced by cyclophosphamide. *Cancer Biochem. Biophys.* **9**: 265–270; 1987.
- [18] Cooper, J. A.; Merrill, W. W. Modulation of endoperoxide product levels and cyclophosphamide-induced injury by glutathione depletion. *J. Appl. Physiol.* **67**:2316–2322; 1989.
- [19] Luderer, U.; Diaz, D.; Faustman, E. M.; Kavanagh, T. J. Localization of glutamate cysteine ligase subunit mRNA within the rat ovary and relationship to follicular atresia. *Mol. Reprod. Dev.* **65**:254–261; 2003.
- [20] National Research Council. *Guide for the care and use of laboratory animals*. Washington, DC: National Research Council, National Academy of Sciences; 1996.
- [21] Armstrong, D. T. Hormonal control of uterine lumen fluid retention in the rat. *Am. J. Physiol.* **214**:764–771; 1968.
- [22] Griffith, O. W. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106**:207–212; 1980.
- [23] Plowchalk, D. R.; Smith, B. J.; Mattison, D. R. Assessment of toxicity to the ovary using follicle quantitation and morphometrics. In: Heindel, J. J.; Chapin, R. E., eds. *Female reproductive toxicology*. San Diego: Academic Press; 1993:57–68.
- [24] Pedersen, T.; Peters, H. Proposal for a classification of oocytes in the mouse ovary. *J. Reprod. Fertil.* **17**:555–557; 1968.
- [25] Hirshfield, A. N.; Midgley, A. R. Morphometric analysis of follicular development in the rat. *Biol. Reprod.* **19**:597–605; 1978.
- [26] Hirshfield, A. N. Size-frequency analysis of atresia in cycling rats. *Biol. Reprod.* **38**:1181–1188; 1988.
- [27] Pasternack, B. S.; Shore, R. E. Analysis of dichotomous response data from toxicological experiments involving stable laboratory mouse populations. *Biometrics* **38**:1057–1067; 1982.
- [28] Griffith, O. W. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J. Biol. Chem.* **257**:13704–13712; 1982.
- [29] deVries, C. R.; Freiha, F. S. Hemorrhagic cystitis: a review. *J. Urol.* **143**:1–9; 1990.
- [30] Greenwald, G. S.; Roy, S. K. Follicular development and its control. In: Knobil, E.; Neill, J., eds. *The physiology of reproduction*. New York: Raven Press; 1994:629–724.
- [31] Ataya, K. M.; Valeriote, F. A.; Ramahi-Ataya, A. J. Effect of cyclophosphamide on the immature rat ovary. *Cancer Res.* **49**: 1660–1664; 1989.
- [32] Coppola, S.; Ghibelli, L. GSH extrusion and the mitochondrial pathway of apoptotic signaling. *Biochem. Soc. Trans.* **28**(Part 2): 56–61; 2000.
- [33] Botchkarev, V. A.; Komarova, E. A.; Siebenhaar, F.; Botchkareva, N. V.; Komarov, P. G.; Maurer, M.; Gilchrist, B. A.; Gudkov, A. V. p53 is essential for chemotherapy-induced hair loss. *Cancer Res.* **60**:5002–5006; 2000.
- [34] Blankenberg, F. G.; Naumovski, L.; Tait, J. F.; Post, A. M.; Strauss, H. W. Imaging cyclophosphamide-induced intramedullary apoptosis in rats using ^{99m}Tc -radiolabeled annexin V. *J. Nucl. Med.* **42**:309–316; 2001.
- [35] Cai, L.; Hales, B. F.; Robaire, B. Induction of apoptosis in the germ cells of adult male rats after exposure to cyclophosphamide. *Biol. Reprod.* **56**:1490–1497; 1997.
- [36] Wang, G. J.; Cai, L. Relatively low-dose cyclophosphamide is likely to induce apoptotic cell death in rat thymus through Fas/Fas ligand pathway. *Mutat. Res.* **427**:125–133; 1999.
- [37] Schwartz, P. S.; Waxman, D. J. Cyclophosphamide induces caspase 9-dependent apoptosis in 9L tumor cells. *Mol. Pharmacol.* **60**:1268–1279; 2001.
- [38] Chen, B.; Cyr, D. G.; Hales, B. F. Role of apoptosis in mediating phosphoramidate mustard-induced rat embryo malformations in vitro. *Teratology* **50**:1–12; 1994.
- [39] Mirkes, P. E.; Little, S. A. Cytochrome c release from mitochondria of early postimplantation murine embryos exposed to 4-hydroperoxycyclophosphamide, heat shock, and staurosporine. *Toxicol. Appl. Pharmacol.* **162**:197–206; 2000.
- [40] Little, S. A.; Mirkes, P. E. Teratogen-induced activation of caspase-9 and the mitochondrial apoptotic pathway in early post-implantation mouse embryos. *Toxicol. Appl. Pharmacol.* **181**: 142–151; 2002.
- [41] Huang, C.; Hales, B. F. Role of caspases in murine limb bud cell death induced by 4-hydroperoxycyclophosphamide, an activated analog of cyclophosphamide. *Teratology* **66**:288–299; 2002.
- [42] Celli, A.; Que, F. G.; Gores, G. J.; LaRusso, N. F. Glutathione depletion is associated with decreased Bcl-2 expression and increased apoptosis in cholangiocytes. *Am. J. Physiol.* **275**: G749–G757; 1998.
- [43] Higuchi, Y.; Matsukawa, S. Glutathione depletion induces giant DNA and high-molecular-weight DNA fragmentation associated with apoptosis through lipid peroxidation and protein kinase C activation in C6 glioma cells. *Arch. Biochem. Biophys.* **363**: 33–42; 1999.
- [44] Merad-Boudia, M.; Nicole, A.; Santiard-Baron, D.; Saill, C.; Ceballos-Picot, I. Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson's disease. *Biochem. Pharmacol.* **56**:645–655; 1998.
- [45] Schnellr, T.; Gansauge, S.; Gansauge, F.; Schlosser, S.; Begler, H. G.; Nussler, A. K. Glutathione depletion causes cell growth inhibition and enhanced apoptosis in pancreatic cancer cells. *Cancer* **89**:1440–1447; 2000.
- [46] Wright, S. C.; Wang, H.; Wei, Q. S.; Kinder, D. H.; Larrick, J. W. Bcl-2-mediated resistance to apoptosis is associated with glutathione-induced inhibition of AP24 activation of nuclear DNA fragmentation. *Cancer Res.* **58**:5570–5576; 1998.

- [47] Haouzi, D.; Lekehal, M.; Tinel, M.; Vadrot, N.; Caussanel, L.; Letteron, P.; Moreau, A.; Feldmann, G.; Fau, D.; Pessayre, D. Prolonged, but not acute, glutathione depletion promotes Fas-mediated mitochondrial permeability transition and apoptosis in mice. *Hepatology* **33**:1181–1188; 2001.
- [48] Freeman, M. E. The neuroendocrine control of the ovarian cycle of the rat. In: Knobil, E.; Neill, J., eds. *The physiology of reproduction*. New York: Raven Press; 1994:613–658.
- [49] Mulcahy, R. T.; Wartman, M. A.; Bailey, H. H.; Gipp, J. J. Constitutive and β -naphthoflavone-induced expression of the human γ -glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J. Biol. Chem.* **272**:7445–7454; 1997.
- [50] Cai, J.; Huang, Z.-Z.; Lu, S. C. Differential regulation of γ -glutamylcysteine synthetase heavy and light subunit gene expression. *Biochem. J.* **326**:167–172; 1997.
- [51] Ishikawa, T.; Bao, J.-J.; Yamane, Y.; Akimaru, K.; Frindrich, K.; Wright, C. D.; Kuo, M. T. Coordinated induction of MRP/GS-X pump and γ -glutamylcysteine synthetase by heavy metals in human leukemia cells. *J. Biol. Chem.* **271**:14981–14988; 1996.
- [52] Bailey, H. H.; Gipp, J. J.; Ripple, M.; Wilding, G.; Mulcahy, R. T. Increase in gamma-glutamylcysteine synthetase activity and steady-state messenger RNA levels in melphalan-resistant DU-145 human prostate carcinoma cells expressing elevated glutathione levels. *Cancer Res.* **52**:5115–5118; 1992.