

# Doxorubicin Affects Testicular Lipids with Long-Chain (C<sub>18</sub>-C<sub>22</sub>) and Very Long-Chain (C<sub>24</sub>-C<sub>32</sub>) Polyunsaturated Fatty Acids

Samanta R. Zanetti, Eduardo N. Maldonado, and Marta I. Aveldaño

Instituto de Investigaciones Bioquímicas de Bahía Blanca, Consejo Nacional de Investigaciones Científicas y Técnicas y Universidad Nacional del Sur, Bahía Blanca, Argentina

## Abstract

**Doxorubicin disrupts spermatogenesis by causing apoptosis of spermatogonia and primary spermatocytes. The aim of this study was to examine the effect of this agent on adult rat testicular lipids and their fatty acids. A single dose (7.5 mg/kg) and a multidose regime (3 mg/kg once a week for 4 weeks) were evaluated. Both treatments resulted in the gradual loss of spermatogenic cells and determined a marked reduction in testicular size and weight 9 weeks after their start. Germ cell loss was accompanied by a decrease in phospholipids, including glycerophospholipids and sphingomyelin. Concomitantly, glycerophospholipids lost selectively their major polyunsaturated fatty acid (PUFA), 22:5*n*-6, and sphingomyelin lost its major very long-chain PUFA (VLCPUFA), 28:4*n*-6 and 30:5*n*-6. The molecular species from which the lost polyenes originated were thus a trait of germ cells. A transient peak of 16:0-ceramide was observed 48 h after the single dose. In both doxorubicin regimes, sphingomyelin and ceramide with reduced amounts of VLCPUFA after about 4 weeks and with no VLCPUFA after 9 weeks resulted. By contrast, triglycerides and especially cholesterol esters (CE) tended to accumulate in the testes undergoing germ cell death, probably in the surviving Sertoli cells, their fatty acid patterns suggesting that initially, these lipids retained part of the PUFA coming from, or no longer used for, the synthesis of germ cell glycerophospholipids. As the latter decreased, CE accumulated massively 9 weeks after starting doxorubicin treatment, 20:4*n*-6 becoming their major PUFA. Part of these CEs may derive from surviving steroidogenic cells. [Cancer Res 2007;67(14):6973-80]**

## Introduction

The anthracycline antibiotic doxorubicin, or Adriamycin, is the antineoplastic drug of choice in the treatment of many solid tumors, although one of its adverse effects is male infertility (1-3). In response to several chemotherapeutic agents including anthracyclines, the number of male germ cells undergoing apoptosis increases several fold (4). Because the cells most sensitive to doxorubicin are the early spermatogenic cells, spermatogonia type A and meiotically dividing primary spermatocytes (4, 5), doxorubicin treatment may lead to the loss of proliferating nonmature germ cells and eventually of mature spermatozoa. Although it is

known that anthracyclines interfere with a number of biochemical and biological functions within eukaryotic cells, the precise mechanisms responsible for its adverse actions on the testis have not been completely elucidated. Knowledge of the biochemical factors contributing to germ cell death and related epiphenomena is important because it may help find ways of attenuating germ cell injury without compromising the antitumor efficacy of doxorubicin. For example, free radical formation and lipid peroxidation is known to be involved in the antiproliferative/cytotoxic actions of doxorubicin on tumor cells (6, 7). These processes also occur in the testis of doxorubicin-treated animals, explaining the high vulnerability of proliferating germ cells and pointing to pretreatment with antioxidants as a promising form of reducing doxorubicin toxicity (8-10).

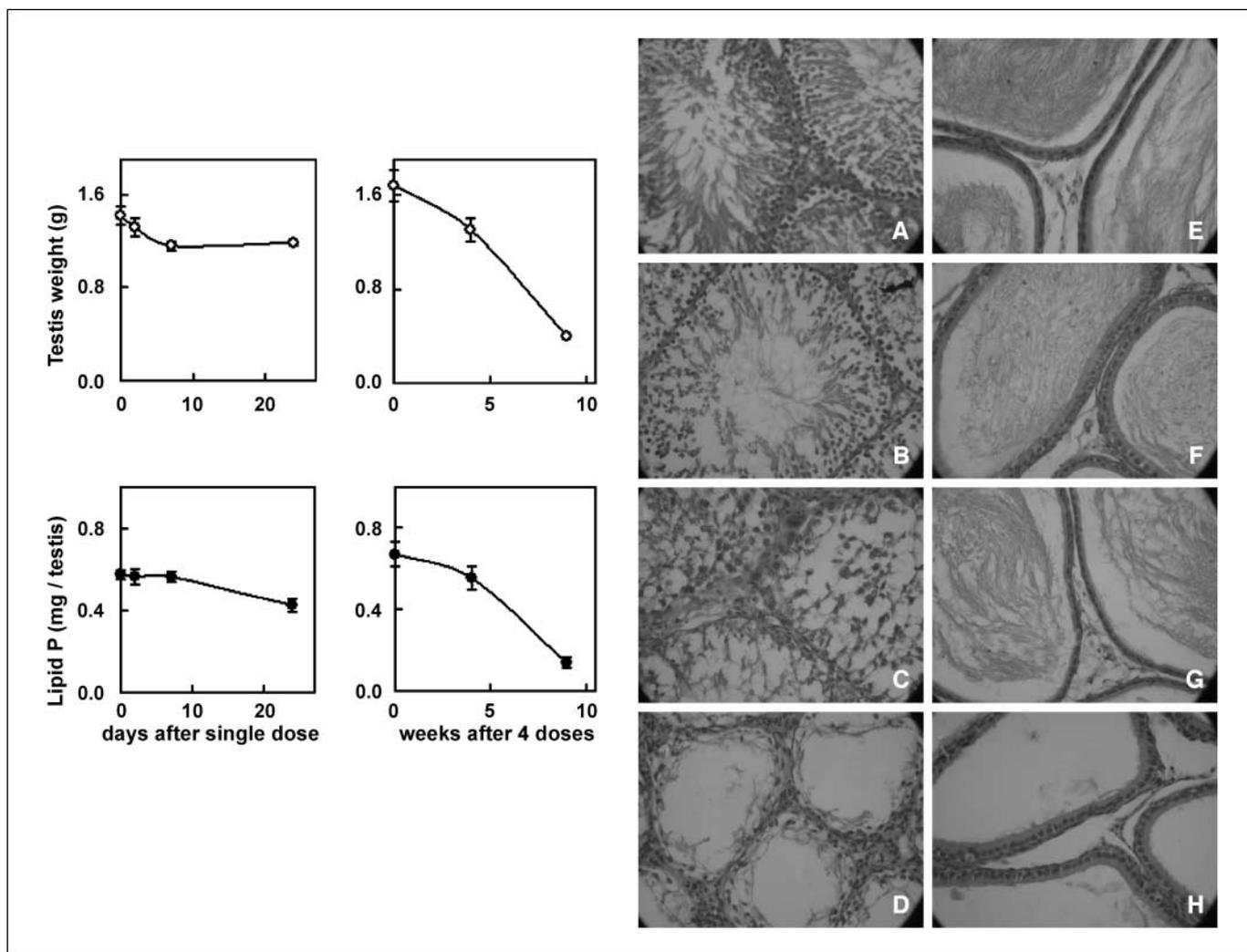
The lipids of adult mammalian testis have long been known to contain high proportions of long-chain polyunsaturated fatty acids (PUFA). In the rat, the most abundant PUFA of testicular glycerophospholipids has long been known to be docosapentaenoic acid (22:5*n*-6), an acyl chain that is a major acyl group of cells of the germ line (11), although it seems to be mostly synthesized by Sertoli cells (12). Besides the well-known PUFA of C<sub>18</sub> to C<sub>22</sub> carbon atoms, the mammalian testis contains in their seminiferous tubules a group of very long-chain PUFA (VLCPUFA) as components of specific lipids (13, 14). The neutral lipids triacylglycerols, 1-alkyl diacylglycerols (A-DAG) and cholesterol esters (CE) contain, in addition to important proportions of 22:5*n*-6, VLCPUFA with 24 to 32 carbon atoms (15). More recently, we investigated the fatty acids of sphingomyelin and ceramide of rat seminiferous tubules (16). In addition to the long-chain saturated fatty acids these sphingolipids have in most tissues and cells, they contain VLCPUFA up to C<sub>32</sub>, 28:4*n*-6 and 30:5*n*-6 prevailing in both (16). The possible changes induced by doxorubicin on the highly unsaturated lipids of testis have not yet been studied in detail. One of the aims of this study was to survey these effects to find clues as to the biochemical changes taking place in the cells that die and in those that survive, in the testis of living animals treated with the drug.

Ceramide is known to be formed from cell membrane sphingomyelin by specific sphingomyelinases in response to a variety of stress-inducing factors that result in apoptotic cell death, among which several antineoplastic drugs are counted (17-20). Because ceramide increases in germ cells undergoing apoptosis in seminiferous tubules *in vitro* (21) and because treatments with doxorubicin induce apoptotic death of specific spermatogenic cells *in vivo* (4, 5), one of the first questions was how doxorubicin treatment would affect the ceramide and sphingomyelin of testis and their fatty acids. However, we soon realized that doxorubicin produced several quantitative and qualitative effects on virtually all testicular lipids of the rat. A few of these were early changes, whereas others could be ascribed to the long-term consequences of the initial death of doxorubicin-sensitive germ cells. We report here that, concomitantly with the progress of testicular involution and

**Requests for reprints:** Marta I. Aveldaño, Instituto de Investigaciones Bioquímicas de Bahía Blanca, Consejo Nacional de Investigaciones Científicas y Técnicas y Universidad Nacional del Sur, La Carrindanga km 7, 8000 Bahía Blanca, Argentina. Phone: 54-291-4861666; Fax: 54-291-4861200; E-mail: avelda@criba.edu.ar.

©2007 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-07-0376



**Figure 1.** Changes in weight and total lipid phosphorus content in rat testis after a single dose (7.5 mg/kg) and after four doses, given once a week (3 mg/kg), of doxorubicin. *Right*, photomicrographs of H&E sections of testis and ductus epididymidis are compared. The mentioned tissues were obtained from control rats, from rats 24 d after a single dose of doxorubicin, and from rats 28 d and 9 wks after the four doses of doxorubicin, in that order. A-D, testis; E-H, epididymis, respectively.

atrophy, large changes in lipid amounts as well as in their PUFA and VLCPUFA profiles occur in rat testis, especially in the period between 4 and 9 weeks after starting doxorubicin treatment.

## Materials and Methods

**Animals and doxorubicin treatments.** Adult male Wistar rats 3 to 4 months old were housed under standard conditions with food and water ad libitum. The protocols for animal experimentation were reviewed and approved by a local institutional Animal Care and Use Committee. Doxorubicin solution was given i.p. (7.5 mg/kg in the single-dose study and 3 mg/kg once a week for 4 consecutive weeks in the multidose regimen). Control rats were injected i.p. with normal saline following the same regimes as the doxorubicin-treated groups. At scheduled points in time, animals anesthetized with ketamine/acepromazine were weighed, operated, and sacrificed immediately after testis removal. Testes and epididymides were fixed in formaldehyde solution for histologic examination. The tissues were embedded in paraffin and stained with H&E.

**Lipid separation and analysis.** The testes were weighed, decapsulated, and rinsed in normal saline after removal of visible blood vessels. Lipid extracts were prepared according to the procedure of Bligh and Dyer (22)

and kept under  $N_2$ . Aliquots from the extracts were taken for total lipid phosphorus determination (23). The phospholipids were resolved into classes on high-performance TLC plates using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by volume; ref. 24). After TLC, lipid zones were located by exposing the plates to iodine vapors, and the phospholipids were quantified by phosphorus analysis of spots (23).

Neutral lipids were resolved by TLC on 500- $\mu$ m-thick silica gel G plates in two steps, using chloroform/methanol/ammonia (90:10:0.2, by volume) up to the middle and then hexane/ether (80:20, by volume) up to the top of the TLC plates. The bands at the origin of these plates were collected to elute the phospholipids. Aliquots of these eluates were taken for phospholipid and fatty acid composition analyses and for the preparative isolation of sphingomyelin and choline glycerophospholipids (CGP). Sphingomyelin was isolated using chloroform/methanol/acetic acid/0.15 mol/L NaCl (50:25:8:2.5, by volume; ref. 25). The CGP were resolved by two-dimensional TLC (23).

The lipid zones were located by exposing the TLC plates to UV light after spraying (using  $N_2$ ) 2',7'-dichlorofluorescein in methanol. All lipids were eluted by transferring the silica support into glass tubes and thoroughly mixing with chloroform/methanol/acetic acid/water (50:39:1:10, by volume), followed by centrifugation. The procedure was repeated thrice (26), followed by partition with 1 N ammonia and drying under  $N_2$ .

The eluted sphingomyelin and ceramide were taken to dryness and treated (under  $N_2$ ) with 0.5 N NaOH in anhydrous methanol at 50°C for 10 min to remove any potential lipid contaminant with ester-bound fatty acids. After alkaline treatment, 1 volume each of chloroform and 0.5 N HCl was added to the methanol solution; the organic phase was rapidly recovered and dried, and the lipids were separated again by TLC.

**Fatty acid analysis.** The lipid fatty acids were analyzed by gas chromatography after derivatization into fatty acid methyl esters. These were prepared from lipids with 2%  $H_2SO_4$  (27) in previously degassed, HPLC-grade methanol (overnight at 45°C) in screw-capped sealed tubes, under  $N_2$ . Methyl heneicosanoate was added as an internal standard for quantitative analysis. Before analysis, the fatty acid methyl ester samples containing the standard were purified by TLC using hexane/ether (95:5, by volume) on silica gel G plates that had been previously washed (with methanol/ethyl ether, 75:25, by volume). The zones were located, the silica support was scraped into tubes, and methyl esters were recovered by thoroughly mixing with methanol/water/hexane (1:1:1, by volume), doing three successive hexane extractions and centrifugations and combining the hexane phases. The VLCPUFA or rat testicular lipids were identified as detailed in previous work (14, 16).

A Varian 3700 gas chromatograph and a Varian Star Chromatography Workstation (version 4.51) were used. The instrument was equipped with two (2 mm × 2 m) glass columns packed with 15% SP 2330 on Chromosorb WAW 100/120 (Supelco, Inc.) and two flame ionization detectors, operated in the dual-differential mode. The column oven temperature was programmed from 150 to 230°C at a rate of 5°C/min, the final temperature being held for 30 min to allow VLCPUFA to emerge from the column. Injector and detector temperatures were 220 and 230°C, respectively, and  $N_2$  (30 mL/min) was the carrier gas.

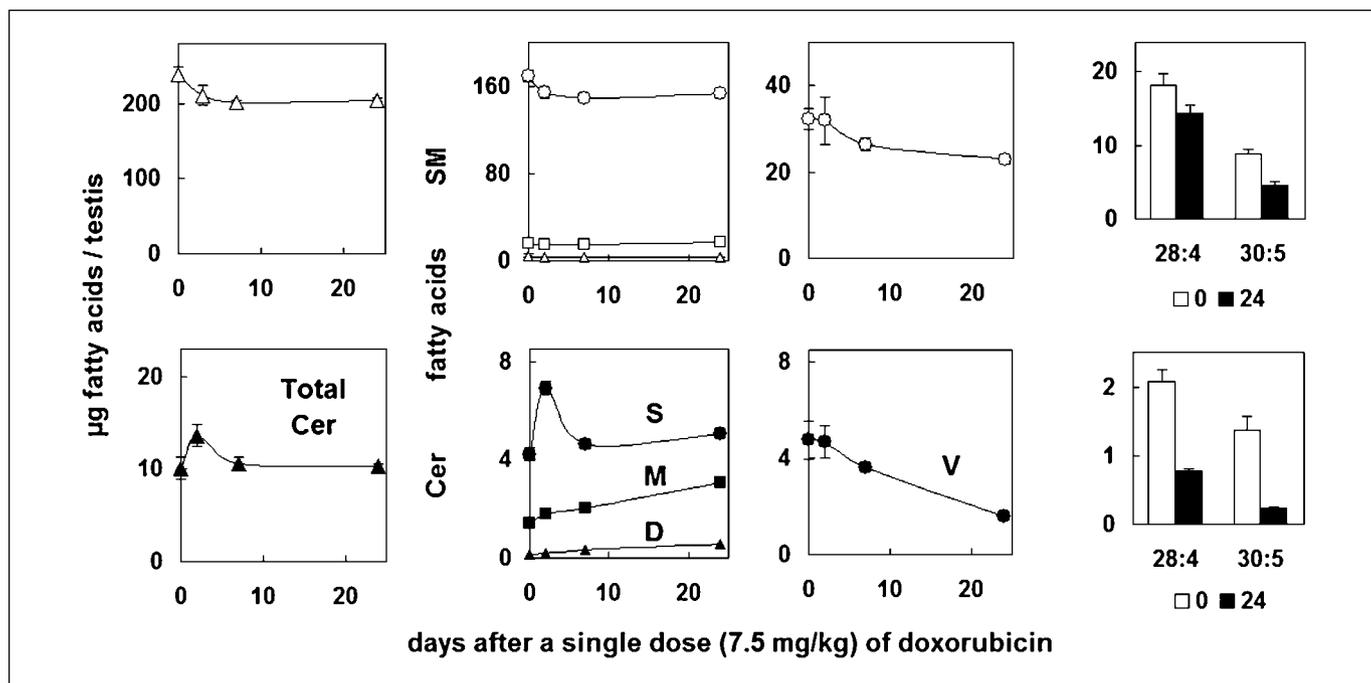
The amounts of lipids in the figures are represented by the sum of the amounts of their fatty acids, directly, with no further corrections or calculations. Also, for simplicity in the presentation of the fatty acid data, in most figures, the lipid fatty acids have been grouped into saturates, monoenes, dienes, polyunsaturates, and very long-chain polyunsaturates, and symbolized by the letters S, M, D, P, and V, respectively.

**Statistical analyses.** All values shown represent mean values from at least three rats per sample  $\pm$ SD. The significance of differences was assayed

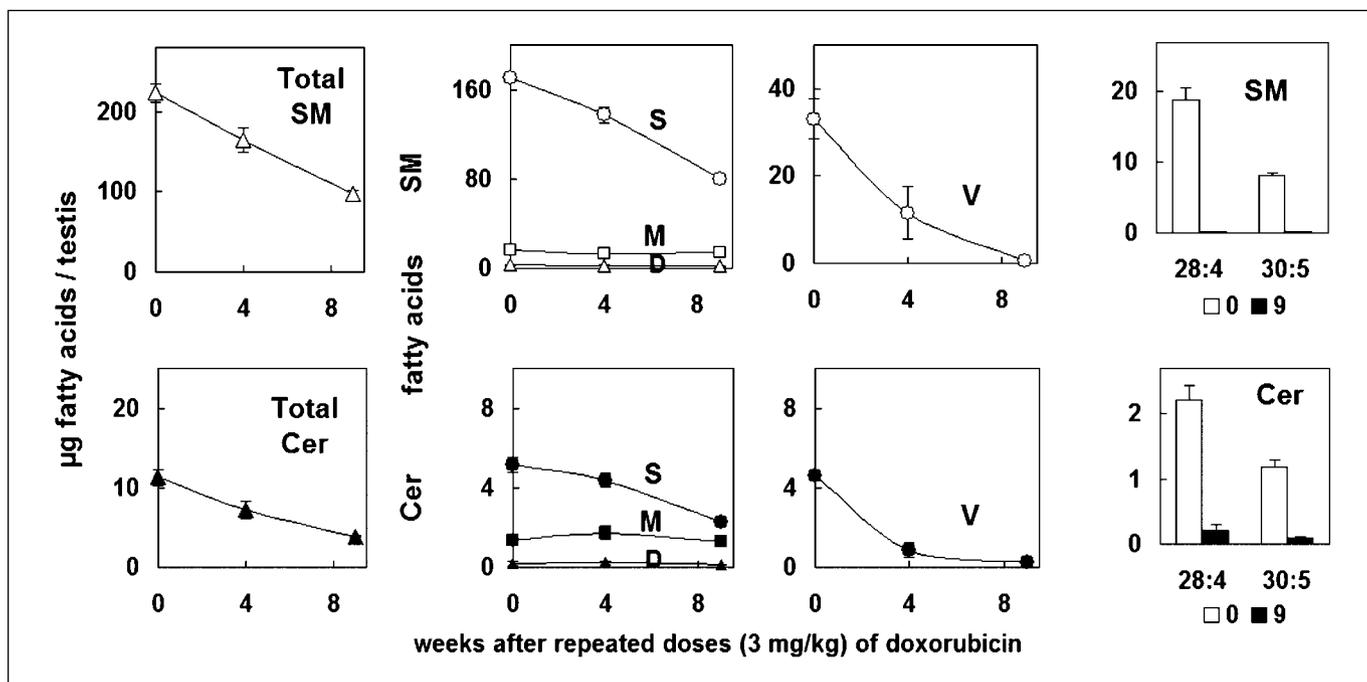
by the two-tailed Student's *t* test ( $P < 0.05$  was considered significant). All differences with respect to controls observed 9 weeks after starting the multidose administration of doxorubicin were statistically significant.

## Results

**Body and testicular weights and histologic examination.** A significant decrease in body weight gain and food consumption was observed in animals treated with doxorubicin in comparison with their respective control groups. On average, the increase in body weight after 9 weeks was 50% in controls and 0% in treated animals (data not shown). The testicular weight and size decreased significantly in the doxorubicin-treated groups (Fig. 1). Twenty-four days after the administration of the single dose, and 28 days after the first of the four weekly repeated doses, the weight of the testis decreased by 17% to 20%. Nine weeks after starting either dose regime (data not shown) or the multidose doxorubicin regime (Fig. 1), the testicular weight was reduced by 73% to 76%, the testes weighing just one-fourth of those of the age-matched control rats. The testicular weight changes were consistent with the main histologic findings, also shown in Fig. 1. In agreement with previous work (28), 24 days after a single dose, there was a moderate but already noticeable loss of germ cells, the depleted cell types included spermatogonia and spermatocytes from preleptotene to pachytene (Fig. 1). A similar result was observed 28 days after starting the multidose regimen, according to previous observations (29). Nine weeks after starting the doxorubicin treatment, marked seminiferous tubule atrophy was observed: the diameter of the seminiferous tubules and the thickness of the germ cell layer were markedly smaller than in the control groups. Sertoli and interstitial cells were the main surviving cells eventually populating the flattened tubules. In the epididymis, the changes observed indicated severe oligospermia after 4 weeks and azoospermia after 9 weeks (Fig. 1). The infertility of rats 9 weeks after the multidose



**Figure 2.** Time course of the changes in the fatty acids of rat testicular sphingomyelin (SM) and ceramide (Cer) after a single dose (7.5 mg/kg) of doxorubicin. The groups of fatty acids of both sphingolipids are expressed by their sums, as follows: S, saturates, M, monoenes, D, dienes, V, polyunsaturated fatty acids. Right, changes in the amount of two VLCPUFA typical of sphingomyelin and ceramide.



**Figure 3.** Long-term effects of doxorubicin on the fatty acids of rat testicular sphingomyelin and ceramide after four doses of doxorubicin (3 mg/kg), repeated once a week for 4 successive weeks. Other details are as in Fig. 2.

doxorubicin regimen was checked by mating the treated males with normal females for 14 successive nights. Although copulations occurred, no pregnancies resulted.

Taken together, the above findings suggested that after almost 1 month, the effects of doxorubicin had started, but had not reached completion. Because doxorubicin treatment destroys stem cells and spermatogonia but spares more of the most differentiated cells of the germ line, such as spermatocytes and spermatids (4, 5), the eventual long-term consequences of germ cell death on the testis required a longer period than a few weeks to reach completion. Because a whole spermatogenic cycle takes 63 days in the rat, sufficient time had to be allowed for the preexisting unaffected spermatogenic cells to have the chance to exit from the testis. This did indeed happen 9 weeks after starting doxorubicin treatment.

**Testicular lipid phosphorus.** The total lipid phosphorus content per testis decreased almost in parallel with the testicular weight (Fig. 1). A single dose of doxorubicin resulted, 24 days afterward, in a 24% decrease in the amount of testicular phospholipids (Fig. 1) and 75% decrease after 9 weeks (data not shown). Four once-a-week doses resulted in a 19% decrease in phospholipid content after 28 days and 79% decrease in 9 weeks (Fig. 1). The eventually massive decrease in phospholipids observed after 9 weeks was thus associated with the annihilated population of germ cells within the seminiferous tubules.

The content of lipids in tissues is usually expressed per unit of weight or per unit of protein or DNA. However, considering that the decrease in the weight and volume of the testis in the present study was due to the loss of preexisting cells, obviously, the content of total proteins, total lipids, and total nucleic acids per testis diminished in parallel. Thus, we decided to show the effects of doxorubicin on lipids and fatty acids directly on a "per-testis" basis.

**Sphingomyelin and ceramide.** In adult fertile rat testis, both sphingomyelin and ceramide contained important proportions of

fatty acids with very long chains (VLCPUFA,  $C_{24}$ - $C_{32}$  PUFA), their sum amounting to 13% to 17% of the total fatty acids of sphingomyelin and 36% to 41% of those of ceramide, the rest being the usual saturated, monoenoic, and dienoic fatty acids present in most other tissues and cells. The fatty acids of sphingomyelin and ceramide are represented by their sums in Figs. 2 and 3, where two major VLCPUFA, 28:4*n*-6 and 30:5*n*-6, are also included.

Anthracyclines are expected to activate sphingomyelin hydrolysis and generate ceramide as a trigger of apoptosis (30). In this study, we analyzed the relatively short-term effects of doxorubicin on sphingomyelin and ceramide fatty acids in the animals receiving one single dose of doxorubicin (Fig. 2) and the long-term effects in those receiving the multidose regimen (Fig. 3). A significant increase (35%) was observed on day 2 in the content of ceramide (Fig. 2). Part of this ceramide could have been produced from sphingomyelin because this lipid decreased slightly at this time point. Interestingly, the peak of ceramide at 48 h was mainly accounted for by its saturated fatty acids (Fig. 2) and within this group, mostly by 16:0. The total VLCPUFA typical of ceramide apparently did not increase at these early stages. On the contrary, they tended to decrease more than other fatty acids in both sphingomyelin and ceramide. Twenty-four days after the single dose, VLCPUFA were the fatty acids that decreased the most in both sphingolipids.

In the multidose regimen, the amounts of testicular sphingomyelin and ceramide decreased significantly 4 weeks after the first dose and continued to decrease thereafter (Fig. 3). The VLCPUFA were the protagonists of this dramatic decrease because the sphingomyelin and ceramide that remained in the testis 9 weeks after starting the doxorubicin treatment virtually lacked VLCPUFA. The selective loss of VLCPUFA from both lipids thus correlated with the selective loss of spermatogenic cells from the seminiferous tubules.

**Glycerophospholipids.** Phospholipid analysis (Fig. 4) showed that all phospholipid classes tended to decrease, although to different extents, after doxorubicin treatments, with EGP and PS decreasing relatively more than CGP and sphingomyelin (Fig. 4). Total phospholipid or total glycerophospholipid fatty acid amounts and composition were determined by taking aliquots of the total phospholipid fraction and subjecting them to acid- or to alkali-catalyzed methanolysis, respectively. Both forms of studying the fatty acids gave essentially similar results, the differences being minimal because the predominant component of the phospholipid fraction were the glycerophospholipids (Fig. 4). In total phospholipids, as in total glycerophospholipids (Fig. 5), PUFA were the predominant fatty acids. The major constituents of the PUFA fraction were 22:5*n*-6 and 20:4*n*-6, and the main components of the VLCPUFA group were 24:4*n*-6 and 24:5*n*-6 (Fig. 4).

Twenty-four days after a single injection and 28 days after four repeated injections of doxorubicin, total phospholipids or glycerophospholipid fatty acids had decreased by 17% to 20%, whereas merely one-fourth of the initial amounts remained after 9 weeks, whether measured on the basis of total lipid phosphorus (Fig. 1) or on the basis of fatty acids (Fig. 5). Thus, the glycerophospholipids, quantitatively more important than any other lipid in the testis, were the lipid constituents most decreased by doxorubicin. All groups of phospholipid fatty acids decreased accompanying these changes approximately at similar rates.

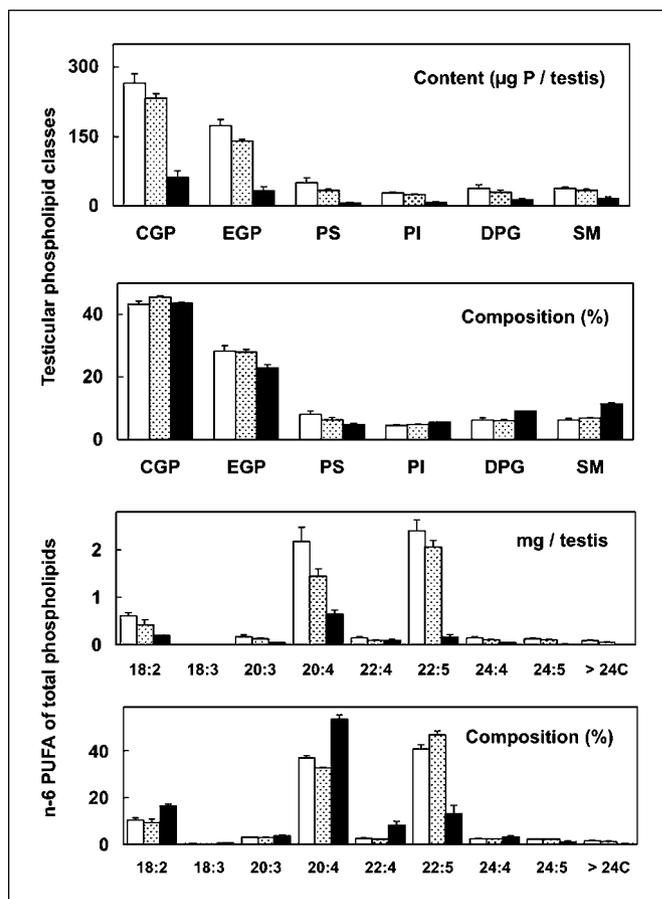
Although the sum of glycerophospholipid-associated PUFA had a similar percentage in control and treated animals, major changes were observed within this group. Thus, 22:5*n*-6 followed by 20:4*n*-6 prevailed throughout the first 24 to 28 days period after doxorubicin treatment, but 22:5*n*-6 decreased much more than 20:4*n*-6 in the period between 4 and 9 weeks (Figs. 4 and 6). Thus, the phospholipids present 9 weeks after doxorubicin had lost most of their 22:5*n*-6 and 24:5*n*-6, becoming instead relatively richer in tetraenoic fatty acids, especially 20:4*n*-6, and in even shorter and less unsaturated polyenes, notably 18:2*n*-6.

**Neutral glycerides and CEs.** In adult fertile rat testis, these neutral lipids were characterized by having important proportions of PUFA (mostly 22:5) and VLCPUFA ( $C_{24}$ - $C_{32}$  tetraenes and pentaenes). These polyenes are represented by their sums in Figs. 5 and 6. In agreement with previous work (15), the main VLCPUFA of triacylglycerols (TAG) were 24:4*n*-6 and 24:5*n*-6; A-DAGs were made up by important proportions of these  $C_{24}$  polyenes and also longer pentaenes and tetraenes up to 32:5*n*-6; the CEs in turn had small percentages of 24 carbon PUFA but large proportions of the longest VLCPUFA, especially 28:5*n*-6, followed by 30:5*n*-6.

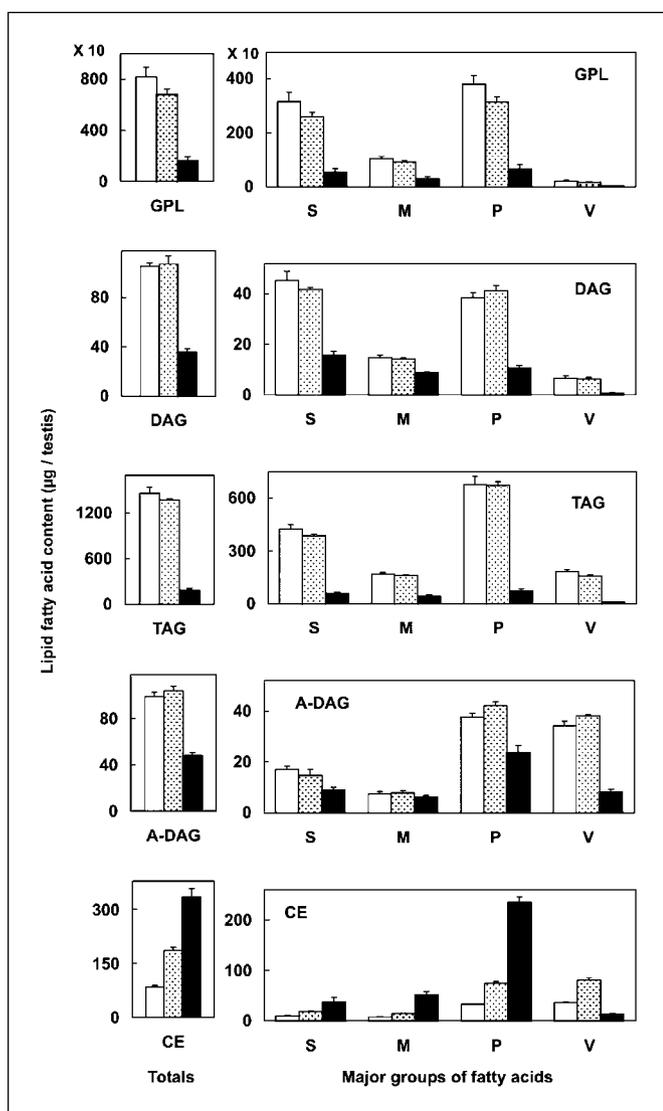
In contrast to glycerophospholipids, the amounts of triglycerides (TAG and A-DAG) per testis were similar as in controls 24 days after a single dose (data not shown) and 28 days after starting the multiple injections of doxorubicin (Fig. 5). Considering that the tissue weight and lipid P decreased, this means that the concentration per gram of tissue or per unit of total lipid P of these neutral lipids increased during the first 4 weeks after doxorubicin administration. Interestingly, this was concomitant with a change in fatty acid profiles. The PUFA and VLCPUFA increased more than did other fatty acids in these neutral lipids, suggesting that (a) they were no longer being used for their normal functions, and/or (b) they were serving as a reservoir of fatty acids, collecting those that originally were in the glycerophospholipids of the dying spermatogenic cells. Nine weeks after the weekly repeated doxorubicin doses, the amounts per testis of TAG and A-DAG were reduced to 20% and 50% of their initial levels,

respectively. Although on a percentage basis, the major acyl groups of triglycerides continued to be PUFA and VLCPUFA (Fig. 5), after 9 weeks, both these lipids had lost most of their original 22:5*n*-6 and longer pentaenes, their profile resembling the picture shown for glycerophospholipids in Fig. 4.

In the testis of doxorubicin-treated animals, CE was the only lipid class to undergo an early, marked, and sustained trend of increase in both amount and concentration. Thus, 9 weeks after starting the doxorubicin treatment, CE had become one of the most important lipid classes of rat testis (Fig. 5). A dual effect was noted in the amounts of PUFA and VLCPUFA of testicular CE: both tended to build up after 3 to 4 weeks, whereas after 9 weeks, the fatty acids mostly responsible for CE accumulation ceased to be these, being replaced mostly by shorter PUFA with less double bonds (notably 20:4*n*-6, but also 22:4*n*-6, and even 18:2*n*-6; Fig. 6). The right of this figure shows that the major PUFA of CE tended to change in a similar direction to that of the PUFA of the main phospholipid class, CGP. It is interesting to note that the CGP of the cells populating the testis 4 weeks after starting doxorubicin administration had a somewhat higher 22:5*n*-6/20:4*n*-6 ratio than the controls, completely contrary to the situation 5 weeks later.



**Figure 4.** Decrease in content and change in the composition of testicular phospholipids in rats that received four doses of doxorubicin (3 mg/kg). White bars, controls; gray and black bars, doxorubicin treated, after 4 and 9 wks, respectively. The phospholipid classes depicted are CGP, choline; EGP, ethanolamine glycerophospholipids, respectively; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol; SM, sphingomyelin. Bottom, content of *n*-6 PUFA in the total glycerophospholipids under the same three conditions, and the percentage of the *n*-6 PUFA composing that group (normalized to 100%).



**Figure 5.** Reduction in the amount of lipid fatty acids in rats that received four doses of doxorubicin (3 mg/kg). White bars, controls; gray and black bars, doxorubicin-treated rats, after 4 and 9 wks, respectively. GPL, total glycerophospholipids; DAG, diacylglycerols; TAG, triacylglycerols; A-DAG, alkyl-diacylglycerols.

## Discussion

The results presented here show that treatments with doxorubicin cause an important decrease in testicular weight by reducing substantially the spermatogenic cell population. An expected but still striking doxorubicin effect was the notorious decrease in the amount of the major testicular lipid components, the phospholipids, including glycerophospholipids and sphingomyelin. The eventual loss of glycerophospholipids was associated with a significant exhaustion of their main PUFA components, in particular 22:5*n*-6, in agreement with this fatty acid being mostly a germ cell-specific glycerophospholipid fatty acid (13). Coincidentally, a substantial loss of sphingomyelin concurred with the disappearance from the testis of its typical VLCPUFA.

From the beginning of the doxorubicin treatments, the VLCPUFA of sphingomyelin and ceramide decreased significantly after 3 to 4 weeks (Fig. 2) and almost completely disappeared after

9 weeks (Fig. 3). This was consistent with the histologic observations (Fig. 1) showing partial and total loss, respectively, of cells of the spermatogenic line at these time points. Because eventually, only somatic cells survive, the present results support our interpretation that VLCPUFA-rich species of sphingomyelin and ceramide are mainly an attribute of the spermatogenic cell line in the testis. A similarly selective depletion of VLCPUFA-rich sphingomyelin and ceramide was observed in other models of germ cell death, including cryptorchidism, ischemia reperfusion, and X-ray irradiation (16). This idea is also sustained by the fact that normal spermatozoa of the rat contain sphingomyelin as well as ceramide with VLCPUFA.<sup>1</sup>

The single injection of 7.5 mg/kg doxorubicin was useful to detect an early change affecting the sphingolipids: a significant increase in ceramide 48 h after this event (Fig. 2). Coincidentally, 48 h after doxorubicin dosing is the reported period necessary to generate the maximum number of apoptotic cells in the seminiferous tubules of adult (5) and immature (31) rat testis. The ceramide-associated fatty acid mostly responsible for this small peak of ceramide was a saturate, 16:0, the ceramide VLCPUFA not changing significantly at that moment. Evidence exists in other non-neural tissues that C-16:0 ceramide represents the ceramide species increasing in amount in apoptosis (32–35). Although indirectly, this supports our idea that VLCPUFA-containing species of testicular ceramide probably are not involved as mediators in proapoptotic mechanisms. This is one of the few cases in which *in vivo* generation of endogenous ceramide is shown in the context of doxorubicin-induced cell death, and one of a few showing a certain degree of specificity toward definite molecular species of ceramide.

All lipids of the germ cell-depleted testis differed greatly in amount and fatty acid composition from their original untreated counterparts. This included diacylglycerols and ceramides whose fatty acids showed similar trends of changes as those of glycerophospholipids and sphingomyelin, respectively, as expected from their metabolic relation. Thus, after 9 weeks of treatment, (a) the content of sphingomyelin and ceramide was low and their major acyl chains were saturated, with a small percentage of monoenoic and dienoic fatty acids; (b) the amounts of glycerophospholipids and triglycerides had decreased several fold, their new major PUFA being 20:4*n*-6 followed by 18:2*n*-6; (c) the only lipids that had increased dramatically, accumulating enormous amounts of 20:4*n*-6 in place of the original PUFA and VLCPUFA, were the CEs. This describes the characteristics of the lipids in the cells that survived doxorubicin effects, among which were Sertoli and interstitial cells, probably also affected by the drug, directly or indirectly.

The CE changes observed here as a consequence of doxorubicin were clearly biphasic: at first, CE, as did triglycerides, tended to accumulate and to concentrate long-chain PUFA (e.g., 22:5*n*-6) and VLCPUFA more than other fatty acids, but in the end, in contrast to TG, CE continued to accumulate, arachidonic acid predominating among its acyl groups. We interpret the first part as these neutral lipids acting as collectors of a fraction of the acyl groups that were being generated from the membranes of the dying cells. Concerning CE, the glycerophospholipid-originated free fatty acids and the free cholesterol located in close contact in apoptotic cell membranes could become esterified in an energy-dependent way,

<sup>1</sup> S. Zanetti et al., 2007, unpublished data.

thereby generating a lipid innocuous to the cell at the same time as saving the elements (cholesterol and PUFA) for future use. Because in the present case, the effects of doxorubicin were irreversible, the testis did not have the opportunity to use these elements further. Over time, CE may have become a redundant lipid class, accumulating merely because these acyl groups had nowhere to go, considering the impressive testicular involution.

The new, unusually enlarged pool of CE observed here as an end result of doxorubicin action obviously accumulated in the cells that survived the effects of the drug. The next question that arises is: in which cells? Our preferred candidates are Sertoli cells, affected by the absence of germ cells. In the new setting (no more spermatogenic cells), they could continue synthesizing fatty acids that would have no membrane glycerophospholipids to end up in. Over time, the genes encoding the desaturases and elongases required to make 22:5 $n$ -6 and longer PUFA would be down-regulated or silenced, the new situation eventually resembling in many respects that of an immature testis. It would be interesting to know whether and at which time points of doxorubicin treatment protein or mRNA levels of the corresponding desaturases or elongases are potentially down-regulated. The second candidates to consider could be Leydig cells. As steroidogenic cells, they store CE in intracellular lipid droplets whose abundance decreases or increases remarkably after administration of hGC or gonadotrophin inhibitors, respectively (36). The first, rate-limiting step in the synthesis of steroid hormones (removal of cholesterol side chain) in steroidogenic cells is known to occur in the mitochondria. Coincidentally, rat testicular mitochondriopathy is another sequel of repeated doxorubicin treatment (37), the reduced activity of two steroidogenic enzymes and the lower levels of serum testosterone also observed (9, 37) being probably secondary to this deleterious

action. Doxorubicin-induced accumulation of CE could thus reflect in part a reduced use of CE as a source of cholesterol for testosterone generation.

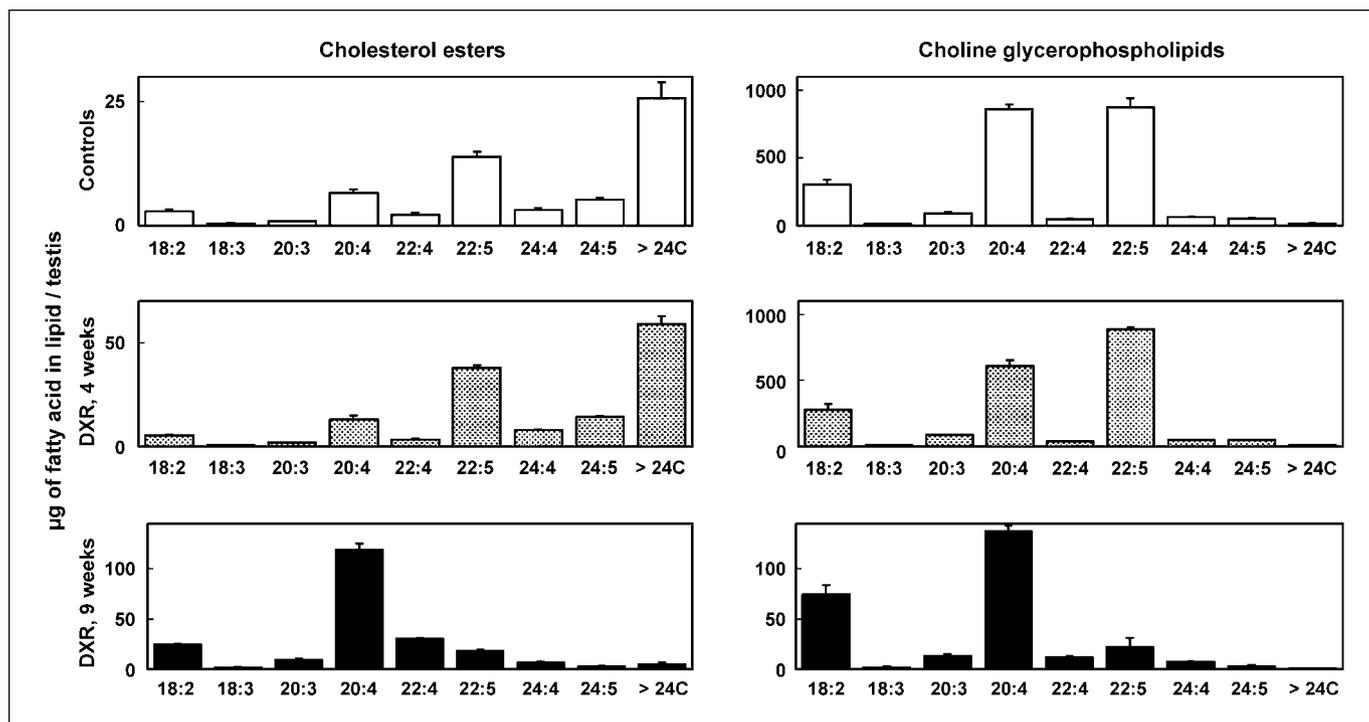
In conclusion, doxorubicin treatment markedly affected the content and fatty acid composition of testicular lipids, including sphingolipids, glycerophospholipids, and neutral lipids. The importance of these lipid changes is that they occur *in vivo*. Early effects on lipids occurred in parallel with the significant damage the drug produced to the germ cells, whereas in the later effects, doxorubicin-surviving cells of the testis were the protagonists. A small peak of ceramide involving 16:0-containing species may indicate a role for this lipid in the triggering of germ cell death. The continuously decreasing 22:5 $n$ -6-rich glycerophospholipids and VLCPUFA-rich sphingomyelin followed the fate of the dying germ cells. Among the surviving cells, Sertoli cells could be responsible for the increased triglycerides and CEs rich in 22:5 $n$ -6 and other pentaenes observed after 4 weeks of treatment, whereas Leydig cells could also be important as contributors of the accumulation of 20:4 $n$ -6-rich CE observed 9 weeks after doxorubicin treatment. Determining whether this accumulation results from a physiologic adaptation to the effects of doxorubicin or reflects just another lipid derangement caused by the drug remains to be investigated.

## Acknowledgments

Received 1/30/2007; revised 4/2/2007; accepted 5/4/2007.

**Grant support:** Consejo Nacional de Investigaciones Científicas y Técnicas, Secretaría General de Ciencia y Tecnología, Universidad Nacional del Sur, y Agencia Nacional para la Promoción de la Ciencia y la Tecnología, Argentina.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.



**Figure 6.** Amounts of PUFA of the  $n$ -6 series in testicular CEs and CGPs in control rats (*top*) and in rats that received four doses of doxorubicin (3 mg/kg). *Middle*, 4 wks after and *bottom*, 9 wks after starting the doxorubicin injections.

## References

1. Shamberger RC, Sheins RJ, Rosenberg SA. The effects of postoperative adjuvant chemotherapy and radiotherapy on testicular function in men undergoing treatment for soft tissue sarcoma. *Cancer* 1981;47:2368-74.
2. Meistrich ML, Goldstein LS, Wyrobek AJ. Long-term infertility and dominant lethal mutations in mice treated with Adriamycin. *Mutat Res* 1985;152:53-65.
3. Imahie H, Adachi T, Nakagawa Y, Nagasaki T, Yamamura T, Hori M. Effects of Adriamycin, an anticancer drug showing testicular toxicity, on fertility in male rats. *J Toxicol Sci* 1995;20:183-93.
4. Sjöblom T, West A, Lähdetie J. Apoptotic response of spermatogenic cells to the germ cell mutagens etoposide, Adriamycin, and diepoxybutane. *Environ Mol Mutagen* 1998;31:133-48.
5. Shinoda K, Mitsumori K, Yasuhara K, et al. Doxorubicin induces male germ cell apoptosis in rats. *Arch Toxicol* 1999;73:274-81.
6. Bachur NR, Gordon SL, Gee MV. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res* 1978;38:1745-50.
7. Sinha BK. Free radicals in anticancer drug pharmacology. *Chem Biol Interact* 1989;69:293-317.
8. Park ES, Kim SD, Lee MH, et al. Protective effects of *N*-acetylcysteine and selenium against doxorubicin toxicity in rats. *J Vet Sci* 2003;4:129-36.
9. Atessahin A, Turk G, Karahan I, Yilmaz S, Ceribasi AO, Bulmus O. Lycopene prevents Adriamycin-induced testicular toxicity in rats. *Fertil Steril* 2006;85:1216-22.
10. Prahalathan C, Selvakumar E, Varalakshmi P. Modulatory role of lipoic acid on Adriamycin-induced testicular injury. *Chem Biol Interact* 2006;160:108-14.
11. Beckman JK, Coniglio JG. A comparative study of the lipid composition of isolated rat Sertoli and germinal cells. *Lipids* 1979;14:262-7.
12. Saether T, Tran TN, Rootwelt H, Christophersen O, Haugen TB. Expression and regulation of  $\Delta 5$ -desaturase,  $\Delta 6$ -desaturase, stearyl-coenzyme A (CoA) desaturase 1, and stearyl-CoA desaturase 2 in rat testis. *Biol Reprod* 2003;69:117-24.
13. Robinson BS, Johnson DW, Poulos A. Novel molecular species of sphingomyelin containing 2-hydroxylated polyenoic very-long-chain fatty acids in mammalian testes and spermatozoa. *J Biol Chem* 1992;267:1746-51.
14. Avelaño MI, Robinson BS, Johnson D, Poulos A. Long and very long chain polyunsaturated fatty acids of the *n*-6 series in rat seminiferous tubules. Active desaturation of 24:4*n*-6 to 24:5*n*-6 and concomitant formation of odd and even chain tetraenoic and pentaenoic fatty acids to C32. *J Chem Biol* 1993;268:11663-9.
15. Furland NE, Maldonado EN, Avelaño MI. Very long chain PUFA in murine testicular triglycerides and cholesterol esters. *Lipids* 2003;38:73-8.
16. Furland NE, Zanetti SR, Oresti GM, Maldonado EN, Avelaño MI. Ceramides and sphingomyelins with high proportions of very long chain polyunsaturated fatty acids in mammalian germ cells. *J Biol Chem* 2007 Mar 29 [Epub ahead of print].
17. Levade T, Jaffrezou JP. Signalling sphingomyelinases: Which, where, how and why? *Biochim Biophys Acta* 1999;1438:1-17.
18. Hannun YA, Luberto C. Ceramide in the eukaryotic stress response. *Trends Cell Biol* 2000;10:73-80.
19. Bezombes C, Laurent G, Jaffrezou JP. Implication of raft microdomains in drug induced apoptosis. *Curr Med Chem Anti-Canc Agents* 2003;3:263-70.
20. Dimanche-Boitrel MT, Meurette O, Rebillard A, Lacour S. Role of early plasma membrane events in chemotherapy-induced cell death. *Drug Resist Updat* 2005;8:5-14.
21. Soumalainen L, Hakala JK, Pentikainen V, et al. Sphingosine-1-phosphate in inhibition of male germ cell apoptosis in the human testis. *J Clin Endocrinol Metab* 2003;88:5572-9.
22. Bligh EG, Dyer WJ. A rapid method for total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911-7.
23. Rouser G, Fleischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 1970;5:494-6.
24. Holub BJ, Skeaff CM. Nutritional regulation of cellular phosphatidylinositol. *Methods Enzymol* 1987;141:234-44.
25. Brown ER, Subbaiah PV. Differential effects of eicosapentaenoic acid and docosahexaenoic acid on human skin fibroblasts. *Lipids* 1994;29:825-9.
26. Arvidson GAE. Structural and metabolic heterogeneity of rat liver glycerophosphatides. *Eur J Biochem* 1968;4:478-86.
27. Christie WW. The analysis of fatty acids. In: Christie WW, editor. *Lipid analysis*. 2nd ed. Oxford: Pergamon Press; 1982. p. 63-92.
28. Bobadilla M, Suter L, Bechter R. Flow cytometry as a tool for the evaluation of testicular toxicity. *Adv Exp Med Biol* 1998;444:73-6.
29. Plassmann S, Urwyler H. Improved risk assessment by screening sperm parameters. *Toxicol Lett* 2001;119:157-71.
30. Herr I, Wilhelm D, Bohler T, Angel P, Debatin KM. Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *EMBO J* 1997;16:6200-8.
31. Hou M, Chrysis D, Nurmio M, et al. Doxorubicin induces apoptosis in germ line stem cells in the immature rat testis and amifostine cannot protect against this cytotoxicity. *Cancer Res* 2005;65:9999-10005.
32. Thomas RL, Jr., Matsko CM, Lotze MT, Amoscatto AA. Mass spectrometric identification of increased C16 ceramide levels during apoptosis. *J Biol Chem* 1999;274:30580-8.
33. Kroesen BJ, Pettus B, Luberto C, et al. Induction of apoptosis through B-cell receptor cross-linking occurs via *de novo* generated C16-ceramide and involves mitochondria. *J Biol Chem* 2001;276:13606-14.
34. Kalthorn T, Zager RA. Renal cortical ceramide patterns during ischemic and toxic injury: assessments by HPLC-mass spectrometry. *Am J Physiol* 1999;277:F723-33.
35. Osawa Y, Uchinami H, Bielawski J, Schwabe RF, Hannun YA, Brenner DA. Roles for C16-ceramide and sphingosine 1-phosphate in regulating hepatocyte apoptosis in response to tumor necrosis factor- $\alpha$ . *J Biol Chem* 2005;280:27879-87.
36. Aoki A, Massa EM. Subcellular compartmentation of free and esterified cholesterol in the interstitial cells of the mouse testis. *Cell Tissue Res* 1975;165:49-62.
37. Prahalathan C, Selvakumar E, Varalakshmi P. Lipoic acid ameliorates Adriamycin-induced testicular mitochondrial pathology. *Reprod Toxicol* 2005;20:111-6.