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Abstract: Cadmium is known to harm rat testis by causing the dose-dependent apoptotic or necrotic death of seminiferous epithelium cells. Here we investigated how this affects the lipids with long-chain (C18-C22) and very-long-chain (C24-C32) polyunsaturated fatty acids (VLCPUFA) typical of spermatogenic and Sertoli cells. A severe acute inflammatory reaction resulted from the massive necrotic death of these cells two days after a single high (4 mg/kg) dose of CdCl2. This led to the conversion of most testicular glycerophospholipids to diradylglycerols (DRG) and free fatty acids (FFA) and of most sphingomyelins to ceramides (Cer). By day 30 the testis weight had decreased three fold. The DRG and FFA had been metabolized but, unexpectedly, ceramides persisted. Also slow to disappear were VLCPUFA-containing triacylglycerols from former germ cells and ether-linked triglycerides and cholesteryl esters (CE) from former Sertoli cells. Similar results were observed 30 and 45 days after administering repeated small non pro-inflammatory CdCl2doses (1 mg/kg). At day 30 after both treatments, an amorphous material replaced the original seminiferous tubules and testicular macrophages populated the interstitium. Species of CE and ether-linked triglycerides containing fatty acids other than VLCPUFA steadily accumulated in the irreversibly damaged testis, a manifestation of the activity of phagocytic cells. The long-term permanence of original VLCPUFA-containing neutral lipids, especially ceramides, indicates that these cells were slow to clear out the acellular material contained in seminiferous tubules, pointing to a form of silent chronic inflammation as an additional outcome of the multifactorial commotion caused in the testis by experimentally administered cadmium.

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June 19, 2013

Dr. Suzanne Jackowski
Dear Executive Editor,

We are submitting the manuscript entitled “Long-term biopermanence of ceramides, cholesteryl esters and ether-linked triglycerides with very-long-chain polyunsaturated fatty acids in the cadmium-damaged testis” by Dr. S. R. Zanetti and myself, to be considered for publication in BBA-Molecular and Cell Biology of Lipids.

The lipids of the cells present in the seminiferous tubules of the adult rat testis are so unique that they can be used as biomarkers of biochemical processes that take place under different experimental conditions. For example, in the present study, the massive hydrolysis of germ cell sphingomyelin into ceramide that occurs after the Cd-induced death of germ cells can be recognized because of the unusual very long-chain polyunsaturated fatty acids (e. g., 28:4, 30:5) these two lipids contain. This is also the case of neutral lipids like cholesteryl esters and ether-linked triglycerides, Sertoli cell products that have specific very-long-chain fatty acids (like 28:5 or 24:5, respectively) in addition to the major long-chain PUFA of rat testis lipids, 22:5n-6. The long-term biopermanence of these neutral lipids is remarkable, our results showing that, in contrast to glycerophospholipids, they are slow to be metabolized by the immune cells that populate the testis.

Given the widely known cytotoxic and potentially carcinogenic properties of cadmium, many articles may be found in the literature about its effects on several of its most sensitive target tissues (lung, bone, kidney, heart, liver, or testis), but those focusing on tissue or cell LIPID CLASSES in particular, are really scarce. Regarding the testis, data on Cd effects on testicular polar and neutral lipid classes, all of them measured on the same basis to facilitate comparisons as in the present study, are even scarcer.

The toxicological and pharmacological literatures are generous in studies dealing with the Cd-induced production of reactive oxygen and nitrogen species that *damage* the polyunsaturated fatty acids of testicular lipids (measured as increased levels of TBARS), their focus being mostly placed on the efficacy of a variety of antioxidant substances to reduce or to counteract such generation.

Our results surveying early and late *in vivo* changes of constitutive germ and Sertoli cell lipid classes, especially after the single high Cd dose, shows that the effects of this metal are much more massive, devastating and irreversible than previously recognized.

All material is original, unpublished, and sent exclusively to this journal.

Looking forward to hearing from you, we are grateful for your considering our work.

Yours sincerely,

Dr. Marta I. Aveldaño

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Highlights

Lipids reveal previously unnoticed short- and long-term Cd effects on rat testis cells

A high Cd dose leads to necrosis, inflammation, and massive phospholipid hydrolysis

Yet, at day 30 ceramides, triglycerides (TG) and cholesterol esters CE) remain as such

Similar long-term lipid changes are seen after low, non proinflammatory Cd doses

New CE and ether-linked TG species are formed, pointing to slow phagocytic activity

Long-term biopermanence of ceramides, cholesteryl esters, and ether-linked triglycerides with very-long-chain PUFA in the cadmium-damaged testis

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Testicular lipids after experimental exposures to cadmium

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ABSTRACT

Cadmium is known to harm rat testis by causing the dose-dependent apoptotic or necrotic death of seminiferous epithelium cells. Here we investigated how this affects the lipids with long-chain (C18-C22) and very-long-chain (C24-C32) polyunsaturated fatty acids (VLCPUFA) typical of spermatogenic and Sertoli cells. A severe acute inflammatory reaction resulted from the massive necrotic death of these cells two days after a single high (4 mg/kg) dose of $CdCl_2$. This led to the conversion of most testicular glycerophospholipids to diradylglycerols (DRG) and free fatty acids (FFA) and of most sphingomyelins to ceramides (Cer). By day 30 the testis weight had decreased three fold. The DRG and FFA had been metabolized but, unexpectedly, ceramides persisted. Also slow to disappear were VLCPUFA-containing triacylglycerols from former germ cells and ether-linked triglycerides and cholesteryl esters (CE) from former Sertoli cells. Similar results were observed 30 and 45 days after administering repeated small non pro-inflammatory $CdCl_2$doses (1 mg/kg). At day 30 after both treatments, an amorphous material replaced the original seminiferous tubules and testicular macrophages populated the interstitium. Species of CE and ether-linked triglycerides containing fatty acids other than VLCPUFA steadily accumulated in the irreversibly damaged testis, a manifestation of the activity of phagocytic cells. The long-term permanence of original VLCPUFA-containing neutral lipids, especially ceramides, indicates that these cells were slow to clear out the acellular material contained in seminiferous tubules, pointing to a form of silent chronic inflammation as an additional outcome of the multifactorial commotion caused in the testis by experimentally administered cadmium.

Key words:
germ cells, Sertoli cells, macrophages, sphingomyelins, triacylglycerols, very long chain fatty acids

1. INTRODUCTION

Cadmium is a common environmental and occupational hazard whose adverse effects on male reproductive health are widely recognized. Cd-induced testicular injury results from a complex network of causes: it disrupts the testicular microvasculature, damages the integrity of the seminiferous epithelium by targeting specialized Sertoli-germ cell and inter-Sertoli protein junctions, is an endocrine disruptor, and is an inductor of significant oxidative stress [1]. During cadmium-induced oxidative stress highly reactive and short-lived oxygen and nitrogen species are produced that damage proteins, nucleic acids, and polyunsaturated fatty acids (PUFA) of cell lipids. Malondialdehyde and other thiobarituric acid-reactive substances (TBARS) are typical by-products of PUFA oxidation by these reactive species that have been extensively used in toxicological and pharmacological studies as tools to evaluate the extent of testicular oxidative stress *in vivo* and to assess the potential efficacy of a variety of substances in reducing and/or preventing this oxidation (see for example data in rat [2,3] and mouse [4] testis). Possible effects on the endogenous lipids of the testis have not yet been explored in this context, except for two early reports showing that cadmium administration to rats results in reduced total phospholipid content and decreased percentage of a major PUFA, docosapentaenoic acid (22:5n-6) [5,6].

The present study was designed to evaluate how cadmium exposure affects the level of rat testicular lipid classes that normally contain high levels of molecular species with long-chain (C_{18}-C_{22}) and very-long-chain (C_{24}-C_{32}) PUFA (VLCPUFA). In their non-hydroxylated (N) and 2-hydroxylated (2-OH) versions (see Scheme 1), the latter fatty acids were first described as components of sphingomyelin (SM) [7] and also of novel glycosphingolipids [8] in the rat and mouse testis, respectively. We have previously shown that the endogenous ceramides (Cer) of rat testis and spermatozoa are also exceedingly rich in N- and 2-OH VLCPUFA, mainly C_{28}-C_{32} (n-6) tetraenoic and pentaenoic fatty acids [9]. Our interest in these unique species stemmed from the fact that, just like the major 22:5n-6-rich species of glycerophospholipids (GPL) and triacylglycerols (TAG), these seminiferous epithelium sphingolipids are exclusive components of germ cells (spermatocytes, spermatids) [10]. The other two neutral lipid classes previously reported to collect 22:5n-6 and VLCPUFA in isolated seminiferous tubules from adult rat testis, namely cholesteryl esters (CE)

and the ether-linked triglycerides 1-alkyl,2,3-diacylglycerols (ADG) [11], are mainly Sertoli cell products [10]. Knowing that cadmium damages both Sertoli and germ cells, we wondered how the testis disposes of their PUFA and VLCPUFA-rich lipids *in vivo* after treatments with the metal.

In this connection we anticipated that the effect of cadmium on adult rat testicular lipids would differ from that of cryptorchidism [12], doxorubicin treatment [13], X-ray irradiation [14] and moderate hyperthermia [15]. These four conditions were previously used as experimental tools to induce the death by apoptosis of vulnerable germ cell precursors, resulting in progressive depopulation of germ cells while sparing Sertoli cells in seminiferous tubules. The general outcome after a few weeks was a gradual germ cell egress, with progressive decrease in PUFA- and VLCPUFA-rich membrane lipids including ceramides, and a temporary build-up of 22:5n-6-rich CE and ADG in Sertoli cells. This build-up was consistent with the phagocytic and lipid metabolizing functions the latter cells normally display. However, these energy-requiring functions may be expected to cease in any condition that leads to the death of Sertoli cells.

Parenterally administered cadmium exerts its deleterious effects on the rat testis via necrotic and/or apoptotic mechanisms, depending on the dose. A single dose of 4 mg/kg $CdCl_2$ damages the testicular microvasculature and causes massive ischemia, leading to necrosis of all seminiferous tubule cells, followed a few hours later by an intense acute inflammatory reaction [16]. It was quantified that doses of 2 mg/kg (and larger) lead to more cell deaths by necrosis than by apoptosis in seminiferous tubules, whereas 1 mg/kg (and lower) is not lethal to all seminiferous tubule cells at once, just provoking focal areas of apoptosis a few hours later [17]. This may be related to the fact that cadmium dose-dependently targets the protein junctional complexes that maintain the connections among seminiferous epithelium cells; initially those between Sertoli cells and germ cells, then those between Sertoli cells, and lastly those between Sertoli cells and the basement membrane [18]. Thus, whereas a high Cd dose leads to massive cell detachment and death, the effects of a single 1 mg/kg dose are spermatogenic stage-specific, mostly affecting the last stages of spermiogenesis [19].

The aim of the present study was to assess the consequences on testicular lipids of massive cell death, followed by an inflammatory reaction, as it happens after necrosis induced by high Cd doses, in comparison with the effects of low, non pro-inflammatory doses. Because a single 1 mg/kg dose in our hands did not result in appreciable short- or long-term changes in testicular lipid levels, we decided to repeat this dose at intervals of 4 days in order to provoke a succession of pro-apoptotic events frequent enough to ultimately cause the death of all intra-tubular cells. Our results showed that the outcome of both treatments was similar: a permanent destruction of the cells originally populating the seminiferous tubules and an increased number of cells in the interstitium. While the major membrane GPL originally belonging to spermatogenic or Sertoli cells were readily hydrolyzed and eliminated from the testis, a relatively high part of the original neutral lipids of these cells -including Cer, triglycerides and CE - remained. Over this rather static picture, new species of CE and ADG were actively accumulated, pointing to an activity of the immune cells populating the interstitium.

2. MATERIALS AND METHODS

2.1. Animals and Procedures

Male Wistar rats aged 4 months and housed under standard conditions with food and water *ad libitum* were given cadmium chloride as a single i.p. dose of 4 mg/kg, or as seven s.c. doses of 1 mg/kg each, one dose every four days, covering 28 days. At scheduled points in time, animals were sacrificed under CO_2 for testis removal. The protocols for animal experimentation were approved by an institutional Animal Care and Use Commission. To confirm that Cd-treated rats exhibited the expected alterations in the testis, those of some animals were fixed in formaldehyde and reserved for histological examination. After embedding the fixed organs in paraffin, thin sections were prepared and stained with hematoxilin-eosin. Body weight gain with time (30 or 45 days after commencing the experiments) and food consumption were inferior ($p < 0.05$) in both cadmium-treated groups compared to their age-matched controls. Rats injected with saline and manipulated in a similar manner to the treated groups were used as the corresponding controls of each experimental condition. The histological and lipid results did not differ significantly from those of untreated animals. In all figures, the mean values from all of these controls are shown.

2.2. Lipid class separation

Lipid extracts were prepared from control and treated testes by homogenization with chloroform-methanol [20], water-partitioned, taken to dryness and dissolved in chloroform-methanol. Aliquots were taken for total lipid phosphorus (P) and total lipid fatty acid analyses. The extracts were spotted (as bands) on TLC plates under N_2 for separation into lipid classes. Hexane/ethyl ether/acetone/acetic acid (30:40:20:1, by vol) up to the middle of the plates resolved the ceramides [9] into those containing nonydroxy and 2-hydroxy fatty acids (Scheme 2). These same plates were dried and a second run with hexane/ether/ acetic acid (80:20:1, by vol) up to the top separated CE from triglycerides (triradylglycerols), the latter directly resolving into TAG and ether-linked triglycerides [11] (see Supplementary Information for details). The bands containing free fatty acids (FFA) and diglycerides (diradylglycerols, DRG) and that containing polar lipids -at the origin of the plates- were recovered for further separations. Major GPL classes and SM were separated essentially as previously described [13]. Care was taken to remove any potential lipid contaminant with ester-bound fatty acids from samples of ceramides and SM by exposing them briefly to mild alkali [9], followed by a second TLC.

In some of the samples, the ether-linked triglycerides were observed to be made up by 1-O-alkyl, 2,3-diacyl-sn-glycerols and 1-alk-1´-enyl, 2,3-diacyl-sn-glycerols (the abbreviation ADG includes both). Their separation was achieved, as described earlier for plasmalogens [21], by exposing dried samples of ADG for 1 minute with one volume of 0.5 N HCl in acetonitrile, followed by the addition of one volume of chloroform and one volume of aqueous sodium bicarbonate for neutralization. The chloroform phase was rapidly recovered, dried, and immediately subjected to TLC using hexane/ether mixtures. This separated the (unaffected) 1-O-alkyl, 2,3-diacyl-*sn*-glycerols from the fatty aldehydes *plus* diacylglycerols generated from 1-alk-1´-enyl, 2,3-diacyl-sn-glycerols (see Supplementary Information).

After separation, all lipid classes or subclasses were eluted from the silica support by thoroughly mixing it with water/methanol/chloroform (1:5:5,by vol), followed by partition of eluates with 4.5 volumes of water [20]. After adding appropriate internal standards, dried samples of the separated lipids in screw-capped tubes were dissolved in (N_2-saturated) anhydrous methanol containing 0.5 N H_2SO_4 and warmed at 45ºC overnight under N_2 to obtain the corresponding fatty acid methyl esters (FAME). After methanolysis, all FAME were subjected to TLC using pre-cleaned silica gel G plates and hexane:ether mixtures [9,13]

2.3. Fatty acid analysis

In addition to ordinary saturated, monoenoic and/or C_{18}-C_{22} PUFA, all rat testicular lipids contained different proportions of (nonhydroxy) VLCPUFA, mostly tetraenes and pentaenes with 24 to 32 carbon atoms. Only SM and Cer contained nonhydroxy and 2-hydroxy VLCPUFA, mostly of 28 to 32 carbon atoms [9]. The latter were recovered as 2-OH FAME and converted into *O*-trimethylsilyl (TMS) ethers for their GC analysis [9,14]. The identity of both types of VLCPUFA was established by Poulos and colleagues more than 2 decades ago in testicular and spermatozoal SM of several mammals using a variety of lipid laboratory criteria and techniques that included mass spectrometry (MS) of the fatty acids themselves and of intact SM molecular species [7,22]. The two types of VLCPUFA were also previously characterized by our laboratory in the ceramides of rat testis using standard lipid analytical techniques including MS of intact and hydrogenated VLCPUFA derivatives [14,23]. The 2-OH VLCPUFA (e.g., 2-OH 30:5n-6) were also thoroughly identified by Sandhoff and colleagues in the mouse testis [8] as components of a novel series of glycosphingolipids.

All numerical data represent mean values from at least three different animals ± SD. The experimental samples were compared to the corresponding age-matched controls (cadmium- *vs* saline- injected animals subjected to the same manipulations). All Cd *vs* control lipid analytical procedures were performed in parallel. The significance of the differences in lipid amounts between animals at each time point was assessed using the two-tailed Student´s *t* test. The figures represent the results for each lipid class as they were obtained, as amounts of fatty acids in each lipid per testis, with no further corrections.

3. RESULTS

A similar long-term decrease in testicular weight was observed in animals treated with $CdCl_2$, whether given as a single high (4 mg/kg) i.p. dose or as smaller (1 mg/kg) but repeated and regularly spaced s.c. doses (Fig. 1). Testicular size and weight as well as the diameter of seminiferous tubules decreased by as much as 70% thirty days after the commencement of each treatment. An amorphous acellular material of similar appearance filled the tubules, replacing the cells that had originally populated them. An increased number of cells in the interstitium and an appreciable fibrotic thickening of testes were eventually observed in both cases.

The main difference between the two regimes occurred during the first few days: the 4 mg/kg dose had an acutely pro-inflammatory effect on the testis whereas the 1 mg/kg doses did not, in agreement with previous work in rats [24]. Two days after administration of the high dose, the testes were severely congested, edematous and hemorrhagic, showing a red-purple discoloration. Compared to control testes (Fig. 1A), they showed an increased number of inflammatory cells in the interstitium (Fig. 1B). These changes were consistent with classical studies [16] showing that this dose of cadmium disrupts the inter-endothelial cell junctions in the peritubular vessel network, and causes extravasation of plasma to the interstitium and the formation of dense plugs of blood cells within testicular microvessels, this resulting in testicular ischemia. By 48 h this ischemia had caused the massive death of cells within seminiferous tubules, leading to the predictable severe acute inflammatory reaction. By day 30 no inflammation remained, the outline of tubules was still discernible and the number of cells had increased in the interstitium, where some microcalcifications appeared (Fig. 1C). That the increased interstitial cells were phagocytes, eventually macrophages, was shown in seminal work demonstrating that these cells actively incorporate previously injected carbon particles and endogenous serum albumin and globulins after administration of cadmium to rats [25].

The lack of apparent testicular inflammation after each dose of 1 mg/kg was also in agreement with previous work showing that administration of just one such dose does not damage the testicular vascular endothelium [19], is not lethal to all seminiferous tubule cells but affects cells at specific stages of the spermatogenic cycle [19], and does not lead to any evident histological changes in rat seminiferous tubules or interstitium one week [24,26] or 56 days [26] after administration. It was repetition of this dose at intervals of 4 days, as we did here, what ultimately caused the death of all cadmium-vulnerable cells.

The dramatic decrease in tissue weight with time after both cadmium regimes (Fig. 1) was a direct macroscopic evidence of the activity of testicular phagocytes metabolizing proteins, nucleic acids, and lipids from dead cells. The dystrophic microcalcifications in the interstitium observed at day 30 after both Cd treatments was consistent with a number of these cells *also* having died in this period, some directly from Cd toxicity, some after phagocytosis of part of the Cd-contaminated tissue debris. In contrast to the irreversibly damaged intra-tubular cells, these cells persisted because they are potentially replaceable, either from pre-existing testicular macrophages, which are capable of undergoing mitosis [27], or from new cohorts of monocytes able to be recruited from the circulation [28].

3.1. Glycerophospholipids

Total cell membrane phospholipid content in the form of total lipid phosphorus (P) decreased concomitantly with testicular weight after the single 4 mg/kg Cd dose (Fig. 1), except at day 2, when lipid P had already fallen sharply though testicular weight was maintained, evidently at the expense of the intense inflammatory edema. This early steep drop in lipid P clearly showed that an intense hydrolysis of phospholipids was taking place. Because Sertoli and germ cells together represent more than 85% of the cells in adult rat testes, hydrolysis of their original membrane lipid constituents was a major contributor to this lipid P decrease. At day 30 the testicular weight had decreased to 29% and the lipid phosphorus content per testis to 14%, of their corresponding control values. Similar long-term results on lipid P were observed at day 30 with the regularly spaced 1 mg/kg doses (Fig. 1).

Figures 2-6 show the comparative *amounts* of lipid classes analyzed in terms of their fatty acids, under 5 different conditions: from left to right, the first bar represents the untreated controls, the next two bars depict the early and late effects of the single (4 mg/kg) dose of $CdCl_2$ (days 2 and 30 post-injection, respectively), and the next two bars represent the effects of the repeated 1 mg/kg doses measured at days 30 and 45 after commencement of the administration.

After the single, high Cd dose, the acute (2 days) fall in lipid phosphorus (Fig. 1) was mostly accounted for by the massive and non-specific decrease in the amount of glycerophospholipids (GPL) per testis (Fig. 2). All of the original testicular GPL classes, rich in species with saturated fatty acids (18:0, 16:0) and long-chain PUFA (20:4n-6, 22:5n-6), including ethanolamine, serine, and inositol GPL (not shown), underwent a substantial decrease, as illustrated in Fig 3 for the major choline glycerophospholipids (CGP).

Two days after the 4 mg/g $CdCl_2$ dose, an important part of the decrease in GPL was mirrored by the generation of large amounts of diglycerides (diradylglycerols, DRG) and free fatty acids (FFA) (Fig. 2). The increased DRG closely resembled in composition the original total GPL, both having saturates and 22:5n-6, followed by 20:4n-6, as their major polyenes. The FFAs that accumulated at day 2 were also saturates and 20:4n-6 followed by 22:5n-6 (Fig. 2). This was accompanied by increased levels of lyso-GPL at day 2 (particularly lyso CGP). A form of phospholipase C to produce DRG and a form of phospholipase A to produce FFA from GPL were evidently responsible for these changes.

Although at a much slower rate compared to the first 2 days, the amount of GPL continued to decrease from days 2 to 30 after the high Cd dose (Fig. 2). The DRG and FFA accumulated at day 2 had decreased to control levels by day 30, suggesting further metabolism that may have included fatty acid oxidation. Thirty days after the successive 1 mg/kg $CdCl_2$ doses, the amounts per testis and fatty acid composition of GPL, DRG, and FFA were comparable to those present 30 days after the single 4 mg/kg dose. The level of these lipids was maintained low from days 30 to 45 (Fig. 2).

3.2. Triacylglycerols

The amount of fatty acids from total lipids (Fig. 3) represented a good biological average between major polar lipids that decreased and minor neutral lipids that increased under the conditions of the present study. Triacylglycerols (TAG) increased more than two-fold 48 hours after the 4 mg/g $CdCl_2$ dose.

With the exception of TAG species containing VLCPUFA, endogenous to seminiferous tubules, most of the TAG fatty acids, especially saturates, monoenes and 18:2n-6, increased significantly the second day after this dose (Fig. 3). Because these fatty acids abound in the TAG of rat plasma lipoproteins[29], which are poor in TAG with 20:4n-6 and 22:5n-6 (5% and less than 1% of TAG fatty acids, respectively), part of the increased TAG at day 2 can be attributed to lipids carried to the site by the inflammatory exudate (serum and cells) that infiltrated the testicular interstitium.

Another part of the increased TAG could have been biosynthesized *in situ*. The accumulation of saturates together with 20:4n-6 and 22:5n-6 in TAG (Fig. 3) suggests a relationship between the formation of these TAG and the increased DAG and FFA (Fig. 2). Since at this point there were no live cells remaining within seminiferous tubule (Fig. 1), such biosynthesis most likely corresponds to the cells populating the interstitium.

The build-up of TAG observed at day 2 after 4 mg/kg $CdCl_2$ was temporary, as TAG levels were similar to those of controls by day 30 after injection (Fig. 3). The TAG levels observed at day 30 after the multiple 1 mg/kg injections were similarly low (Fig. 3). From days 30 to 45 after this last treatment the amount per testis of TAG species rich in 22:5n-6 and 24-32 carbon VLCPUFA represented 80% and 70% of the initial values, respectively (Fig. 3), suggesting a slow but unremitting degree of metabolism of part of the TAG pre-existing in seminiferous tubules.

3.3. Cholesteryl esters

The total amount of CE per testis increased significantly with respect to controls 2 days after the 4 mg/kg cadmium injection (Fig. 4) but, in contrast to TAG, continued to increase at day 30, more than two-fold. At day 30 after commencing the 1 mg/kg Cd injections, the amount of CE per testis was as high as this one, and continued to increase between days 30 and 45 post-treatment.

As in the case of TAG, the CE species that increased most after both Cd treatments with respect to controls were saturates, monoenes, 18:2n-6 and 20:4n-6 (Fig. 4). All of these fatty acids, quantitatively minor in the CE of rat testis, continued to increase with time after both treatments, indicating that the new CE were synthesized in live cells of the testis, most likely in those present in the interstitium.

The amount of CE species containing 22:5n-6 increased approximately two-fold at day 2 but did not change much thereafter (Fig. 4). As a group, the initially abundant CE species containing VLCPUFA were the only CE species in the testis not to increase after the two treatments (Fig. 4).

3.4. Ether-linked triglycerides

The subclass of ether-linked triglycerides (ADG, Fig. 4) markedly increased with respect to control values at day 2 after the single 4 mg/kg Cd dose, remaining equally high at day 30. It was also higher than controls at days 30 and 45 after the repeated 1 mg/kg Cd doses (Fig. 4).

The ADG subclass was found to be made up of the previously reported [11] 1-O-alkyl, 2,3-diacyl-sn-glycerols (alkyl-DAG), plus a relatively smaller but non-negligible proportion of 1-alk-1´-enyl, 2,3-diacyl-sn-glycerols (alkenyl-DAG) (mole ratio of about 1: 0.25 in control testes) (see Supplementary Information). Thus, as it happens with GPL, total triglycerides of rat testis are made up of three subclasses (Scheme 2): TAG with fatty acids ester-bound to the 3 positions of the glycerol backbone and ADG with either a fatty alcohol or a fatty aldehyde at *sn-1* and fatty acids esterified at the other 2 positions of the glycerol backbone. The data for ADG in Figs. 4 and 5 represent the sum of these two subclasses.

Their separation allowed the observation that, in control testes, the two ADG subclasses had both negligible 18:2 and low 20:4n-6, and that the smaller 1-alkenyl-DAG had a relatively higher percentage of 22:5n-6 (and lower amount of 24-32 carbon VLCPUFA) than their relatively larger 1-alkyl-DAG counterparts.

After cadmium treatments, the amount of original ADG species that contained VLCPUFA changed little from days 2 to 30 or 45, in contrast to ADG species containing other fatty acids, which increased significantly in the same period. The "new" species of ADG that increased the most after both Cd treatments contained mainly saturated fatty acids, 20:4n-6, and 22:5n-6. Interestingly, the originally smaller total 1-alkenyl-DAG subclass increased relatively more (263%) than the original 1-alkyl-DAG subclass (45%) 30 days after Cd treatments (Supplementary figures).

3.5. VLCPUFA-containing species of TAG, ADG, and CE

A closer look at the amounts of the fatty acids making up the VLCPUFA group (labeled ≥C24 PUFA in Figures 2-4), revealed that in TAG, ADG and CE, the longest components of this group in fact decreased with time (weeks) after both Cd treatments while the "shorter" ones increased (Fig. 5). Thus, the decreases of VLCPUFA were compensated by increases the comparatively shorter but much more abundant 24 carbon polyenes. Because the longest PUFA are minor but specific endogenous components of the depicted neutral lipids, they served to reveal that these lipids had started to be slowly metabolized with time.

3.6. Sphingomyelins and Ceramides

Just as copious DRG were produced from GPL (Fig. 3), a significant hydrolysis of SM into Cer took place 2 days after the 4 mg/kg $CdCl_2$ dose (Fig. 6). Unexpectedly, whereas the amount of DRG had dropped again by day 30, that of the produced ceramides remained as high at day 30 as at day 2. The amounts of all species of Cer were also higher than in controls at day 30 after the multiple 1 mg/kg doses, with no significant modifications between days 30 and 45. Taking into account that the amount of GPL and their hydrolysis products DRG and FFA had declined, these ceramides became a quantitatively significant component of the total residual lipid remaining in the testis for long periods after both Cd treatments.

The results shown in Fig. 6 suggest that an active form of sphingomyelinase (SM phosphodiesterase, SMPD) hydrolyzed into Cer most of the SM originally associated with seminiferous tubule cells. The fact that the level of such Cer was slow to decrease over the weeks indicated that it was not subsequently acted upon by a similarly active ceramidase.

Nonhydroxy and 2-hydroxy VLCPUFA-containing species of Cer evidently derived from the SM and Cer of spermatogenic cells, while a part of the 16:0- and 18:0-rich species were contributed by Sertoli cells[10], all of which died in situ because of cadmium. At day 2 after the 4 mg/kg $CdCl_2$ dose, most of the SM and Cer species that increased were of extra-tubular or extra-testicular origin, as indicated by the fact that they contained monoenoic and dienoic fatty acids that are minority components in endogenous SM and Cer from seminiferous tubules [10]. Part of these lipids may derive from the SM carried to the site i) as components of

the plasma lipoproteins of the inflammatory exudate; ii) as components of the blood cells trapped in the congested vessels (the SM of rat erythrocytes is rich in 24:0 and 24:1); and iii) as components of the extravasated inflammatory cells that infiltrated the testis. At days 2 and 30 after this condition the sum of SM + Cer fatty acids for saturated, monoenoic and dienoic fatty acid groups exceeded the respective sum of SM + Cer in the original controls. This was not the case at days 30 and 45 after the non pro-inflammatory 1 mg/kg doses.

The species of SM with nonhydroxy VLCPUFA were more readily hydrolyzed into Cer than those with 2-OH VLCPUFA (Fig. 6). Furthermore, the Cer with nonhydroxy VLCPUFA slowly decreased as the weeks post-treatments elapsed, whereas those with 2-OH VLCPUFA increased beyond the level one would expect if they had only derived from hydrolysis of the corresponding SMs. This difference can be explained by the 2-OH VLCPUFA-containing glycosphingolipids described by Sandhoff et al. [8] being also partly hydrolyzed into Cer.

4. DISCUSSION

The presented results add the complexity of lipid changes to the intricate network [1] of events that arise from exposure to this metal. The unique set of long-chain and very-long-chain polyenoic fatty acids of rat testis cells served as biomarkers to reveal i) strong catabolic reactions that affected the major GPL, ii) expected catabolic reactions that occurred intriguingly slowly, such as the disappearance of endogenous and SM-derived ceramides; iii) an also indolent catabolism of pre-existing species of TAG, ADG and CE, as shown by their VLCPUFA; and iv) a steady biosynthesis of lipids, as manifested by the new species of CE and ADG with time.

In contrast to the virtual vanishing by day 30 of most of the original GPL and SM, hydrolysis and further metabolism of *neutral* lipids that had originally belonged either to germ cells –as was the case with the VLCPUFA-rich species of TAG and Cer [10]– or to Sertoli cells –as was the situation with the VLCPUFA-rich species of CE and ADG– was not completed one month after Cd-induced damage. Considering the severe loss of testicular weight, the concentration of all these neutral lipids increased substantially if expressed per gram of tissue.

The Cd-induced long-term persistence of the amorphous organic material filling the space originally occupied by cells within seminiferous tubules was not observed under experimental conditions that cause damage to spermatogenic cells but spare Sertoli cells [12][13][14][15] (see also Supplementary material 2). In all these cases, the eventual differentiation and egress from the testis of those germ cells that survive are possible, resulting in "Sertoli cell only", *patent* tubule lumena. In the present case, Sertoli cells were themselves primary targets of $CdCl_2$: phagocytosis of germ cell corpses and seminiferous tubule fluid formation and secretion were absent, this explaining why cell remnants could not progress along the seminiferous tubules for their elimination.

The acute testicular inflammation that occurred during the first 48 h after the high Cd dose is an expected reaction to the ischemic-dependent necrosis of intra-tubular cells that this dose provokes[16]. As in other tissues [30,31], different subsets of macrophages coexist in rodent testis, the two extremes of this continuum being the classically activated M1 and the alternatively activated M2 cells, discernible from one another by the surface antigens and immune mediators they produce [32,33]. The M1 subset, displays a cytotoxic, pro-inflammatory phenotype, is not normally active in healthy testes, protects the testis against infection and allows for typical inflammatory responses [34]. It may be credited for orchestrating the early inflammation observed.

Rat testes, as other organs, produce TNF-α *in vivo* 48 h after Cd treatment, as well as nitric oxide (NO) and highly reactive oxygen species (ROS) [35]. Rather than mastocytes or other resident cells of the testis, activated M1 macrophages and recruited neutrophils are the most likely cell source of these products. The mentioned short-lived pro-oxidant metabolites are known to damage proteins, nucleic acids and lipid PUFA. They are to be blamed for part of the lipids previously belonging to intra-tubular cells being attacked at their highly unsaturated acyl chains, this giving rise to the increased levels of TBARS detected *in vivo* 48 h after a 2 mg/kg dose of $CdCl_2$ in rat testis [2,3]. These cells may be an important source of the phospholipases whose

activity was overtly manifested in the present study by the rapid and massive degradation of testicular GPL and SM.

Albeit retaining phagocytic functions, M2 macrophages are functionally associated with scavenging cell debris, promoting tissue remodelling and repair, and being responsible for immune suppression [30,31]. Normally, the M2 subset prevails in the healthy rodent testis, where its anti-inflammatory/immunosuppressor profile is critical in protecting germ cells and spermatozoa from immune-mediated attack [36]. Rat testicular macrophages in culture do produce high levels of prostaglandins E_2 and $F_{2\alpha}$, which are able to *inhibit* lymphocyte proliferation [37]. The increased production of prostaglandin $F_{2\alpha}$ that occurs in the rat testis 48 h after treatment with a > 2 mg/kg (but not with a < 1mg/kg) dose of $CdCl_2$ [38] could be an early manifestation of these cells increasing in numbers and/or activity trying to put a limit to Cd-induced damage. In this regard, administration of hemin, through its anti-inflammatory, antioxidant, and antiapoptotic effects, has been shown to protect rat testes against Cd-induced reduction in testosterone levels, to compensate deficits in antioxidant defense systems, and to suppress lipid peroxidation in testicular tissue resulting from cadmium administration [39]. Hemin is an inhibitor of heme oxygenase, precisely an enzyme known to be highly expressed in macrophages.

Chronic inflammation may follow acute inflammation or may start insidiously without any overt manifestation of the latter. The transition from acute to chronic inflammation involves an M1 to M2 phenotype change and occurs when the former fails to be resolved due to the persistence of the agent causing the damage or after insuperable interference with normal healing processes. At day 30 after commencing either of the two dose regimes of this study, irrespective of the initial form of cell demise and of the presence or absence of an initial phase of acute inflammation, the Cd-devastated seminiferous tubules were surrounded by a cohort of extra-tubular live and active cells infiltrating the interstitium. This suggests a similar form of silent, chronic inflammation as the common outcome. The M2 macrophages normally prevailing in the testis may have played an important role in resolving the acute inflammation after the single high Cd dose and perhaps in preventing its development after the frequent low Cd doses. Walling off the potentially antigenic organic material (denatured protein, nucleic acids) contained in the damaged seminiferous tubules by anti-inflammatory/immunosuppressor macrophages seems to be a practical way of shielding the generated neo-antigens from exposure to reactive cells of the immune system.

4.1. Glycerophospholipids and triacylglycerols

The proportion of 22:5n-6 in GPL increases and that of 20:4n-6 decreases as germ cells differentiate from pachytene spermatocytes to spermatids and spermatozoa [10]. The fact that after Cd exposure most of the major GPL originally making up the different germ cell types of seminiferous tubule cells were rapidly hydrolyzed *in situ*, releasing massive amounts of FFA and DRG -mostly DAG- containing these PUFA, is a direct evidence that several phospholipases and related hydrolases were non-specifically activated after cell death: GPL→DRG; GPL→phosphatidic acid (PA); PA→DRG; DRG→FFA; GPL→FFA. Some of these hydrolases may be endogenous to former Sertoli and/or germ cells undergoing autolysis, and some may belong to the live cells that infiltrated the interstitium.

Even taking into account the additional amounts of neutral and polar lipids carried to the site in blood cells and plasma in the acute inflammatory exudate, the fatty acid content per testis in the *total lipid* was considerably lower at days 2 and 30 after the high Cd dose. This indicates that the fatty acids arising from GPL and TAG hydrolysis were either exported or beta-oxidized in due course, evidently by day 30, by live and active cells of the testis, mostly testicular phagocytes able to elude the toxic effects of cadmium.

Because these cells are normally capable of hydrolyzing TAG and utilizing their fatty acids, it was intriguing that an important part of the original TAG, those rich in 22:5n-6 and 24-carbon VLCPUFA, remained high at day 30 after both Cd treatments. These unique TAG are formed in healthy spermatids in the last stages of their differentiation, as they reduce their cytoplasmic volume to become spermatozoa [10]. Together with other materials left behind, these TAG are tightly packed in particles known as “residual bodies”. These particles are normally released in areas proximal to the lumen and distal from the basal membrane of seminiferous tubules [10,14] to be readily engulfed, and their contents disposed of, by Sertoli cells. The persistence of these particular species of TAG for a month or more after cadmium treatments suggests that

most of them remained out of the reach of testicular phagocytes, within the stagnant organic material that replaced former tubules.

4.2. Cholesteryl esters and ether-linked triglycerides

Adult rat testis CE and ADG rich in 22:5n-6 and VLCPUFA are synthesized in Sertoli cells. During normal spermatogenesis these cells continuously phagocytize residual bodies and apoptotic bodies from germ cells and metabolize large amounts of the polar and neutral lipids thus incorporated [14]. The VLCPUFA-containing CE and ADG species we recovered after Cd treatments were thus, in part, an unmodified proportion of the original Sertoli cell CE and ADG. In contrast, the new species of CE and ADG that steadily accumulated with time well after cadmium treatments were obviously produced by live cells. Since Cd is toxic *in vitro* to Leydig cells [40], as corroborated by the fact that Cd administration to rats leads to a complete failure of steroidogenesis [3,38], the long-term survival of these cells under the present conditions is highly unlikely. After the disappearance of Leydig and Sertoli cells, interstitial macrophages are good candidates as responsible for the biosynthesis of the increasing CE and ADG species.

Following endocytosis or phagocytosis, CE may derive from materials containing excess free cholesterol and fatty acids, whether taken up from lipoproteins or from membrane lipids resulting from dead cells. After ester bond hydrolysis and conversion of fatty acids into acylCoA, acylation of cholesterol by acylCoA:cholesterol acyl transferase (ACAT) may be expected to occur.

By the same token, DRG derived from (diradyl) GPL after phosporyl-base removal in phagocytized materials may be combined with a spare fatty acid at *sn-3* by a diglyceride acyl transferase (DGAT) to produce the corresponding triradylglycerols, namely TAG, l-O-alkyl DAG, and alk-1-enyl-DAG subclasses. The fact that the latter subclass was detected in larger amounts than in controls 30 days after Cd exposures (see Supplementary Information) points to diglycerides derived from original plasmalogens as their most likely source. Once formed, the ether-linked triglycerides may be temporarily stored in cell cytoplasmic lipid droplets, as is the case of other neutral lipids including TAG or CE.

Apart from an auto-protective function against excessive intracellular accumulation of DRG and FFA in phagocytes during GPL catabolism, which would apply to all three subclasses of triglycerides including TAG, the reason for the existence of three subclasses of triglycerides in cells remains as intriguing as the reason for the occurrence of three subclasses of membrane GPL.

A striking and infrequently recalled attribute of ADG is their high rate of production in a variety of spontaneously arising and transplantable solid animal and human tumors (see [41] and references therein), thus associating these neutral lipids with the increased cell turnover typical of cancer. Although the cell types within tumors in which these neutral lipids accumulate remain to be identified, it is currently accepted that a chronic inflammatory microenvironment of the type cadmium exposure provokes in the testis provides the fuel for solid tumor promotion, mostly facilitated by tumor-infiltrating macrophages that undergo a phenotypic 'switch' to the alternatively activated, M2 phenotype [42]. The notion that ADG could be produced in this particular type of chronic inflammatory cells in tumors is worth investigating in this context.

4.3 Sphingomyelins and Ceramides

A lysosomal and a secretory form of acid sphingomyelinase (SMPD1) originating in a common protein precursor via divergent cellular trafficking have been identified [43]. The ceramides produced in seminiferous tubules of Cd-treated rats after hydrolysis of the corresponding SM may be ascribed in part to autolysis, to be catalyzed by a form of acid sphingomyelinase originally confined in lysosomes of Sertoli and germ cells and also in acrosomes of spermatids and spermatozoa. The importance of lysosomal acid SMase in normal gonadal function has been demonstrated in *smpd1*-null mice, a model of human Niemann-Pick disease [44,45]. These mice progressively accumulate SM as lipid inclusions in their organs including testes, mostly in Sertoli cells, and have reduced fertility because they produce and release structurally and functionally abnormal SM-rich –and Cer-poor– spermatozoa.

In contrast to the massive hydrolysis of testicular SM into Cer under the present experimental conditions, the long-term persistence of high levels of Cer indicates that the ceramidase activity that should have deacylated

this Cer into sphingosine and FFA was low in the cells that had originally populated the seminiferous tubules. Five ceramidases encoded by 5 different genes have been cloned in humans and mice [46], some of which occur in intracellular and in secretory forms. Activity and expression (mRNA) of membrane-bound neutral ceramidase [47] and lysosomal acid ceramidase [48] are high in several mouse tissues but intriguingly, they are barely detectable in the testis. A low level of ceramidase in testis is thus consistent with the present finding that the Cer molecules produced after Cd treatment take a long time to be degraded.

In the healthy state, the numerically lesser but metabolically very active Sertoli cells could be expected to contain a form of ceramidase, as one of their functions is to phagocytize and catabolize residual bodies and apoptotic bodies that do contain SM [10]. This ceramidase could contribute to the limited though not negligible reduction observed with time in the levels of N-VLCPUFA-containing Cer (Fig. 6). The endogenous ceramidase activity in the –much more numerous– germ cells could be low, taking into account that spermatids [10] and spermatozoa [10,49] endogenously contain significant amounts of Cer with 2-OH VLCPUFA. That mature rat spermatozoa have abundant sphingomyelinase but lack ceramidase activity is indicated by the persistence of high Cer/SM ratios and unchanged levels of Cer in gametes that have undergone capacitation and acrosomal reaction *in vitro* [9].

In addition to catabolic phospholipases and lipases, resident testicular macrophages are expected to contain the active sphingomyelinase(s) and ceramidase(s) inherent to any phagocyte. Although these cells have not yet been studied in this context, lung macrophages do display high constitutive levels of mRNA, protein, and activity of acid ceramidase [50]. Assuming that testicular macrophages also do, the long-term persistence of higher than normal Cer/SM ratios in the presence of such ceramidase suggests two possibilities. One is that these cells did not completely phagocytize the material present in seminiferous tubule remnants, even at days 30 or 45, and that the Cer (as well as part of the original TAG, ADG and CE) remained as residual components of such material. The other is that they did phagocytize part of this material, but that intracellularly accumulated lipids decreased or inhibited further phagocytic or hydrolytic activities.

In support of the first idea is the finding that species of SM with nonhydroxy VLCPUFA belong to less mature spermatogenic cells -like pachytene spermatocytes- and those with 2-OH VLCPUFA to spermatids and nascent spermatozoa [10]. Thus after massive cell death, the former lipids are physically closer to the basal membrane of seminiferous tubules, and thereby more readily accessible to extra-tubular phagocytes and their putative sphingomyelinases and ceramidases than the latter, which are more distant as they normally occur in cells facing the seminiferous tubule lumena. This could explain the relatively more active hydrolysis into Cer of the SM species that contain N- than 2-OH VLCPUFA (Fig. 6) and the decrease in Cer with N-VLCPUFA commencing before those with 2-OH VLCPUFA.

In support of the second possibility, a surprisingly similar outcome to the present one in the testis after Cd treatment was previously observed in the lung parenchyma of rats, where cigarette smoke –a toxicant of which is precisely cadmium– induces alveolar epithelial and endothelial cell apoptosis, oxidative stress, and increase in Cer levels [51]. Although all these outcomes are consistent with an increased number of alveolar macrophages, these cells are prevented from achieving the clearance of apoptotic cells [52], largely due to the high Cer levels. It is thus possible that the Cer accumulated in rat testis after Cd exposure could also play a role in moderating the phagocytosis and/or hydrolysis of the residual material.

A persistent neutral lipid-rich central necrotic core surrounded by active macrophages that progressively become anti-inflammatory as they convert into neutral lipid-laden "foam" cells is the familiar description of an atheroma, a tuberculous granuloma and other forms of chronic inflammation. This resembles the picture resulting from the long-term effect of cadmium on the testis. Although the nature of the *lipid* confined in the central residual material typical of these lesions is barely given a second thought, it is worth investigating whether and to what extent it affects the functions of the macrophages that are supposed to eliminate such material, perhaps contributing to the long-term persistence of this type of lesions in the affected tissues.

Human macrophages in culture shift from pro-inflammatory to inflammation-anergic cells as they time-dependently phagocytize lipid-rich materials –like modified lipoproteins– and become lipid-laden "foam cells" [53]; in the process, these cells down-regulate the production of inflammatory cytokines and progressively become less responsive to pro-inflammatory stimuli. Myelin ingestion by human macrophages

functionally converts them into lipid-laden “foamy” macrophages, involved in suppression of inflammation and unable to respond to prototypical inflammatory stimuli [54]. It is then possible that in the present cases as well, time-dependent accumulation of neutral lipids such as CE, ADG and especially Cer occurs in testicular macrophages as they change their phenotypic profile. Further studies are required not just in testis but also in other tissues to elucidate which lipid components of phagocytized materials are capable of regulating macrophage function and the mechanisms involved.

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Supplementary information

1. Supplementary figures with lipid separations including alkyl- and alkenyl- diacylglycerols
2. Supplementary information. Effects of ischemia on rat testis lipids

Abbreviations

ADG, triglycerides with a fatty alcohol or a fatty aldehyde at *sn-1* (1-alkyl, 2,3-diacyl-*sn*-glycerols and 1-alk-1´enyl, 2,3-diacyl-*sn*-glycerols); CE, cholesteryl esters; Cer, ceramides; CGP, choline glycerophospholipids; DRG, diradyl-glycerols (diglycerides with a fatty acid, a fatty aldehyde or a fatty alcohol at *sn*-1 and a fatty acid at *sn*-2); FFA, free fatty acids; GPL, glycerophospholipids; PUFA, polyunsaturated fatty acids; SM, sphingomyelin; TAG, triacylglycerols. The fatty acids of lipids are abbreviated according to convention - number of carbon atoms: number of double bonds, with n-6 referring to the series of PUFA biosynthetically derived from linoleic acid (18:2n-6), such as 20:4n-6 or 22:5n-6. VLCPUFA, very-long-chain (C24-C32 carbon atom) PUFA, composed of nonhydroxy (N) and 2-hydroxy (2-OH) versions.

FIGURE LEGENDS

Fig. 1. Effects of cadmium administration on adult rat testicular histology, weight, and lipid phosphorus content. A representative section of control testes (A) is compared with samples obtained 2 days (B) and 30 days (C) after the animals received a single i.p. dose of 4 mg/kg $CdCl_2$, and 30 days (D) after commencing a regime of seven s.c. doses of 1 mg/kg administered at 4-day intervals. Asterisks indicate the presence of microcalcifications. The lower panels show the time-courses of the decreases in testicular weight and lipid phosphorus (P) from days 2 to 30 after the single dose (circles), and from days 30 to 45 after the repeated doses (triangles) of cadmium.

Fig. 2. Total lipid, triacylglycerol (TAG) and choline glycerophospholipid (CGP) levels in rat testis after cadmium treatment in terms of their fatty acids. This and the following figures show the comparative amounts of fatty acids in lipids under 5 different conditions: the first bar (white) shows the situation in control animals injected with saline; the next two bars (black and hatched) show the early (48 h) and late (30 days) effects, respectively, of a single (4 mg/kg) i.p. dose of $CdCl_2$; and the last two bars (grey and dotted) show the effects measured at days 30 and 45, respectively, after commencing the administration of seven (1 mg/kg) $CdCl_2$ doses at 4-day intervals. The word "Total" under the panels on the left represents the sum of fatty acids in each of the depicted lipids; ≥C24 PUFA is the sum of polyenoic fatty acids having 24 to 32 carbon atoms.

Fig. 3. Total glycerophospholipids (GPL), diradylglycerols (DRG) and free fatty acids (FFA) of rat testis after cadmium treatment. The data are presented as described in the legend toFigure 2. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single i.p. 4 mg/kg $CdCl_2$ injection; grey and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of $CdCl_2$ every 4 days

Fig. 4. Cholesteryl esters (CE) and alkyl plus alk-1´enyl diacylglycerols (ADG) of rat testis after cadmium treatment. The data are presented as described in Figures 2 and 3. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single i.p. 4 mg/kg $CdCl_2$ i. p. injection; grey and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of $CdCl_2$ every 4 days. Note the apparent lack of any significant change in the amount of total VLCPUFA in the two lipids.

Fig. 5. Amount of representative VLCPUFA in rat testicular CE and ADG compared with major TAG. The data are presented as described in Figures 2 to 4. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single i.p. 4 mg/kg $CdCl_2$ i. p. injection; grey and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of $CdCl_2$ every 4 days. The results show that species with 24-carbon PUFA tended to increase while those with longer chain PUFA significantly decreased ($p<0.05$) in ADG and CE.

Fig. 6. Sphingomyelins (SM) and ceramides (Cer) in rat testis after cadmium treatment. The data are presented as in figures 2 to 5. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single i.p. 4 mg/kg CdCl2 injection; grey and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of CdCl2 every 4 days. The amounts of the different groups of fatty acids, including the two main types of VLCPUFA in both lipids, nonhydroxy and 2-hydroxy derivatives (here abbreviated N-V and 2-OH V, respectively), are depicted. Total V represents the sum of both types of VLCPUFA in each lipid.

SCHEME LEGENDS

Scheme 1. Pentaenoic fatty acids that abound in different lipids of the rat testis

Scheme 2. Structure of ceramides (Cer), cholesteryl esters (CE), and the three triglyceride subclasses referred to in the text: triacylglycerols (TAG) and the two ether-linked triglycerides, alkyl- and alkenyl-diacylglycerols (ADG). The R letters represent the remainder of the depicted hydrophobic chains.

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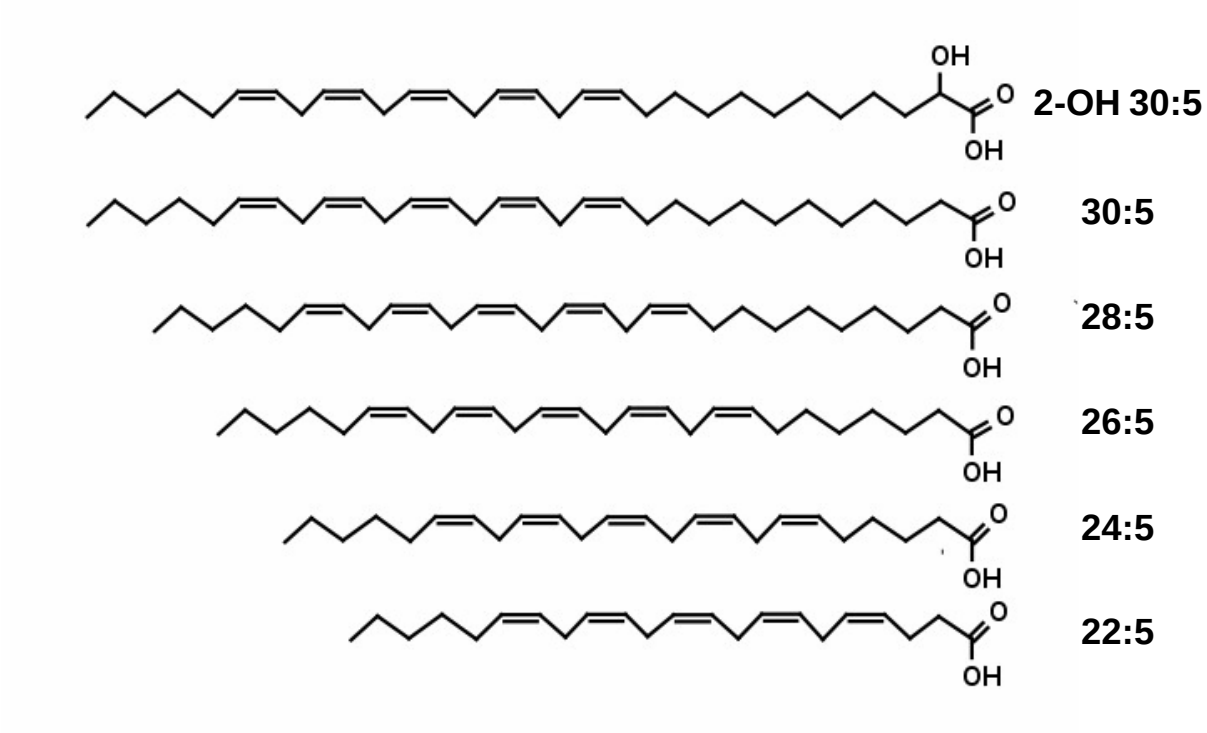
Scheme 1



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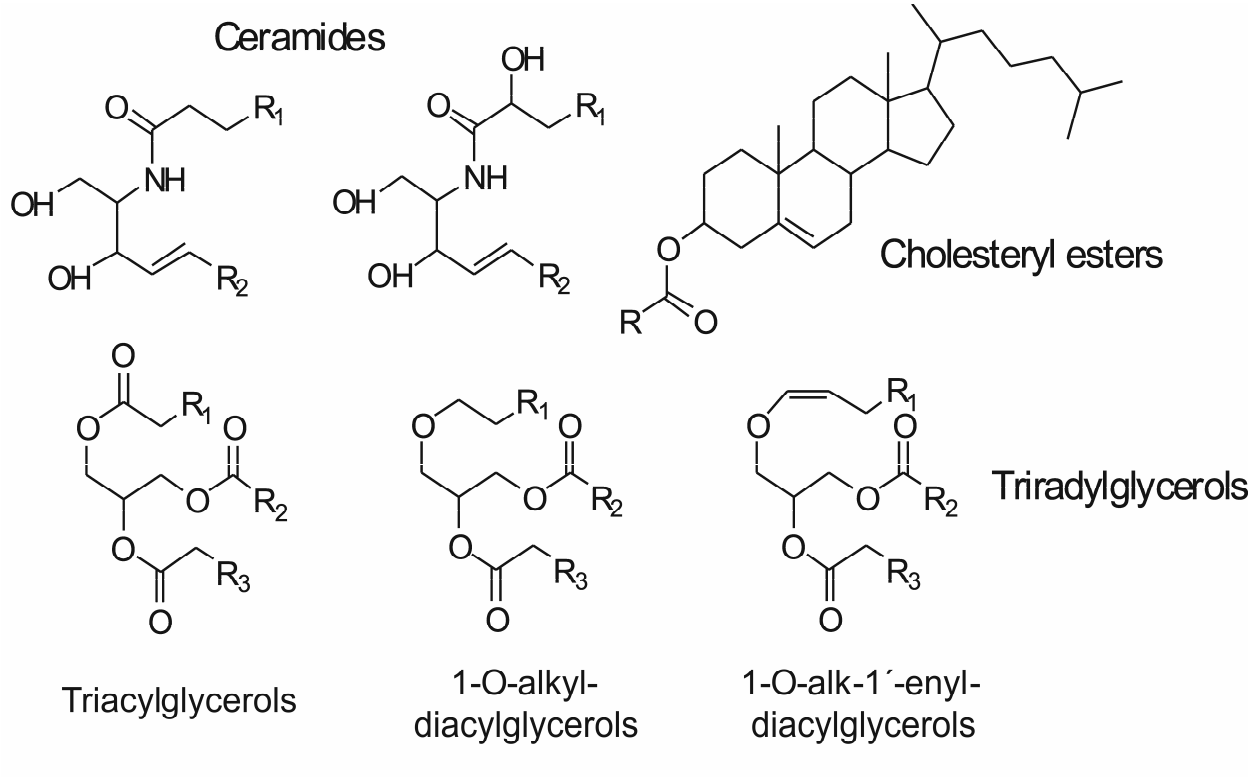

Figure 1

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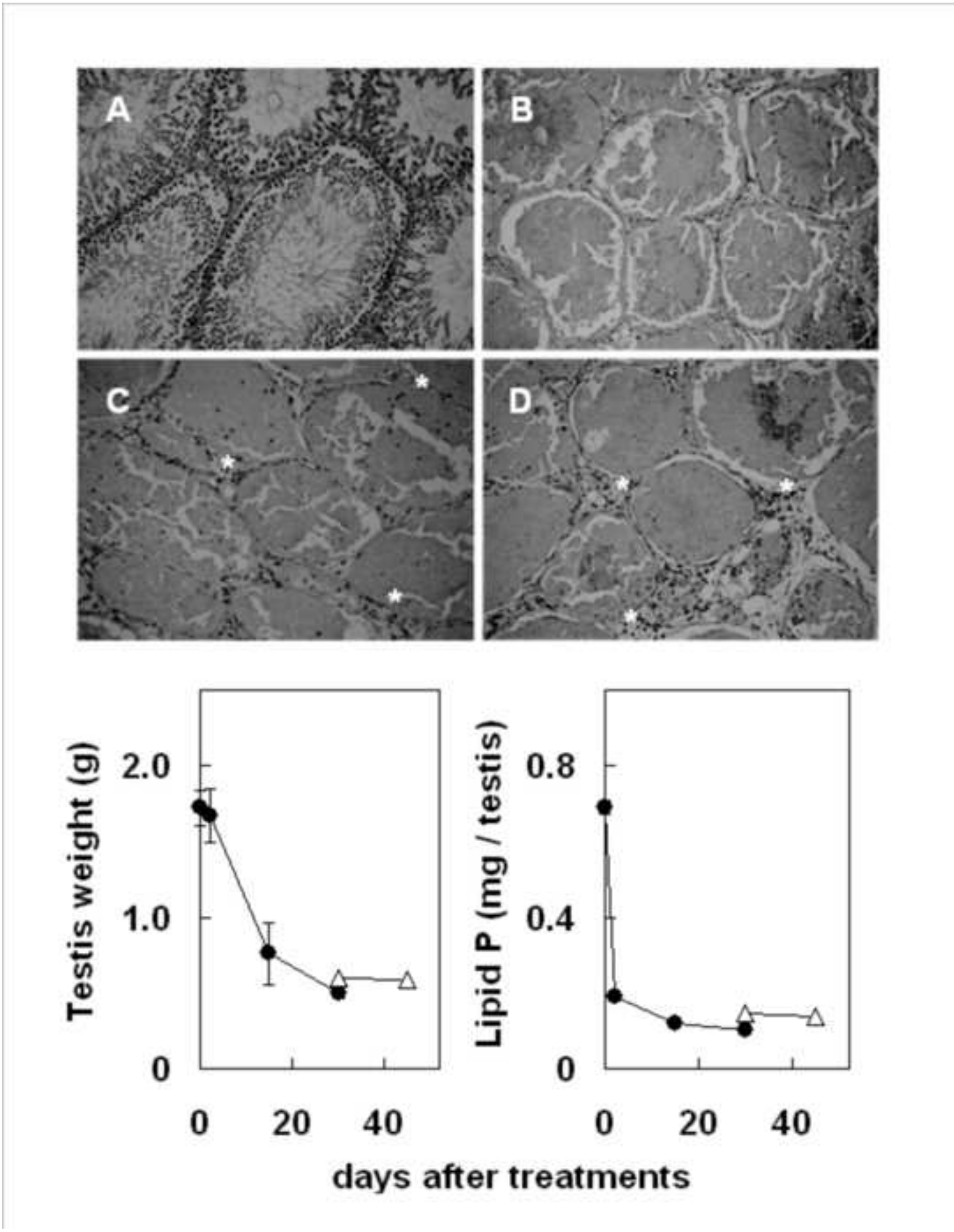

Figure 2

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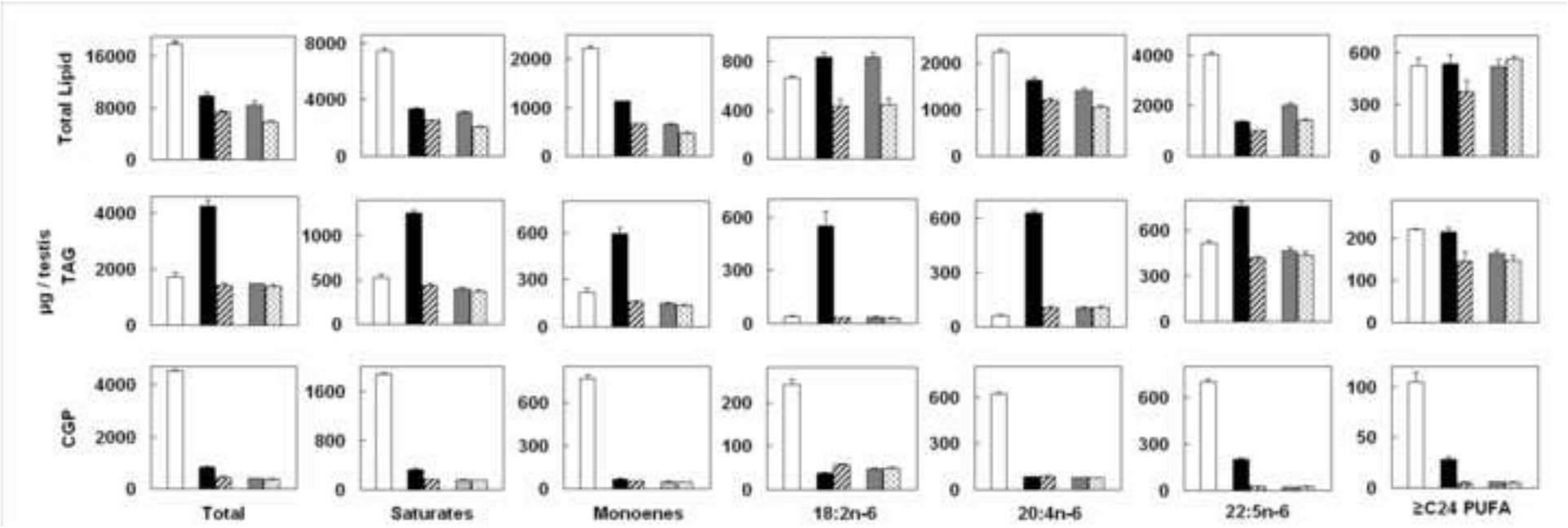

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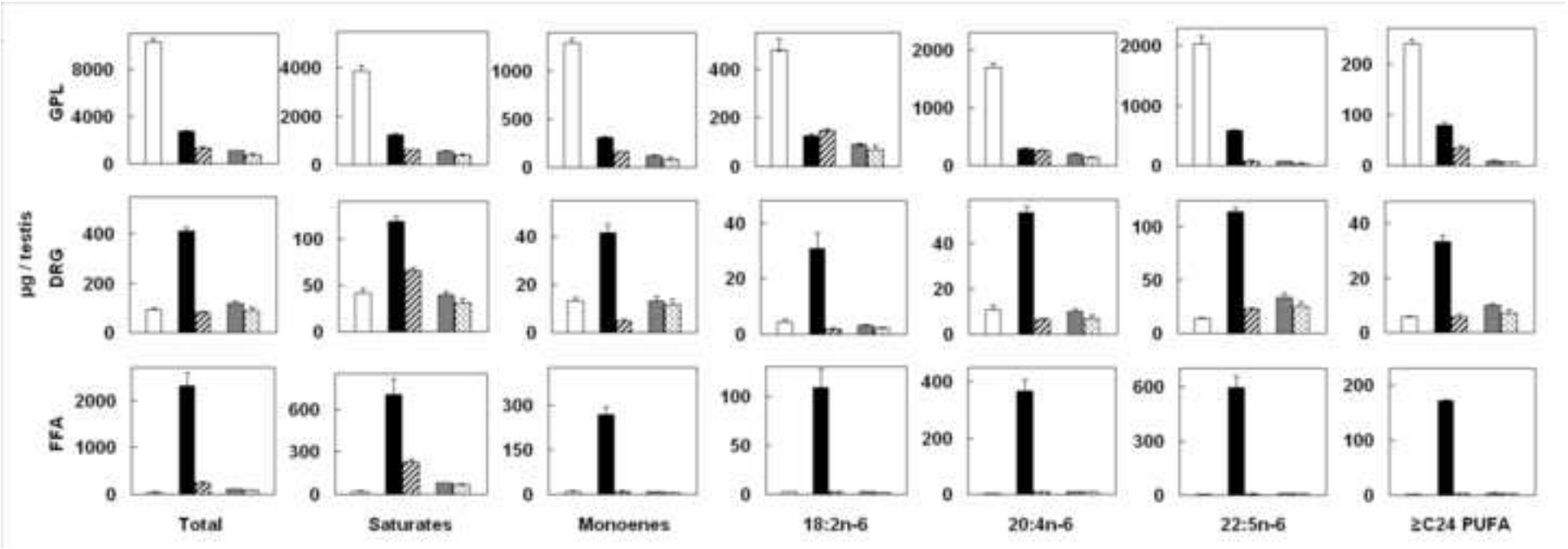

Figure 4

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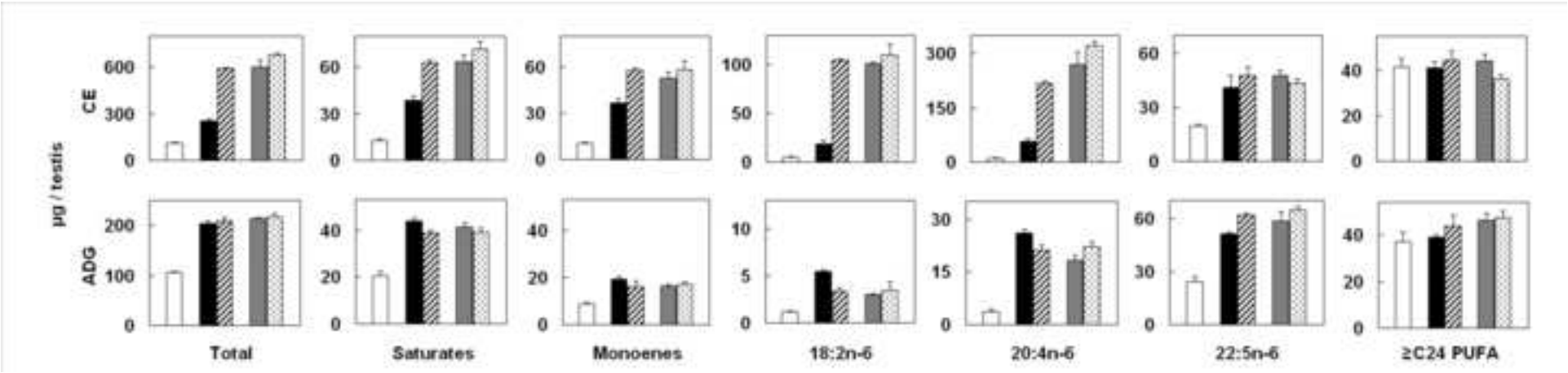

Figure 5

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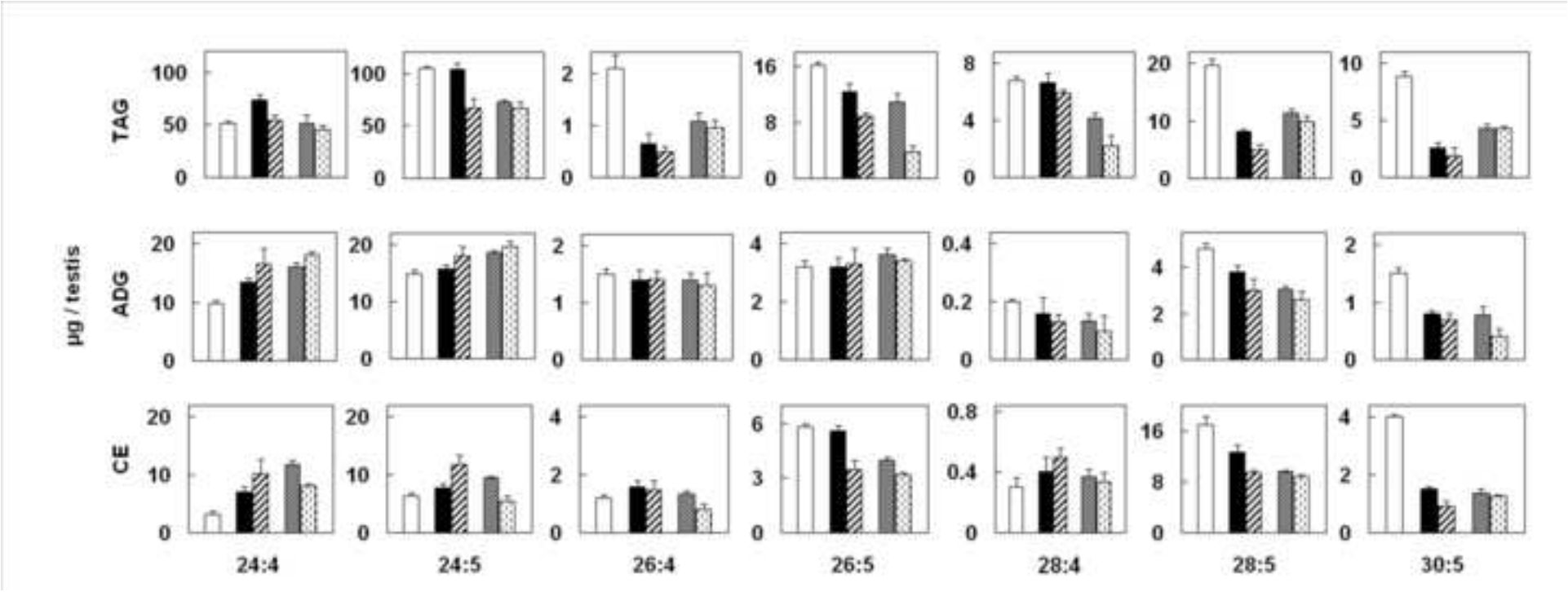

Figure 6

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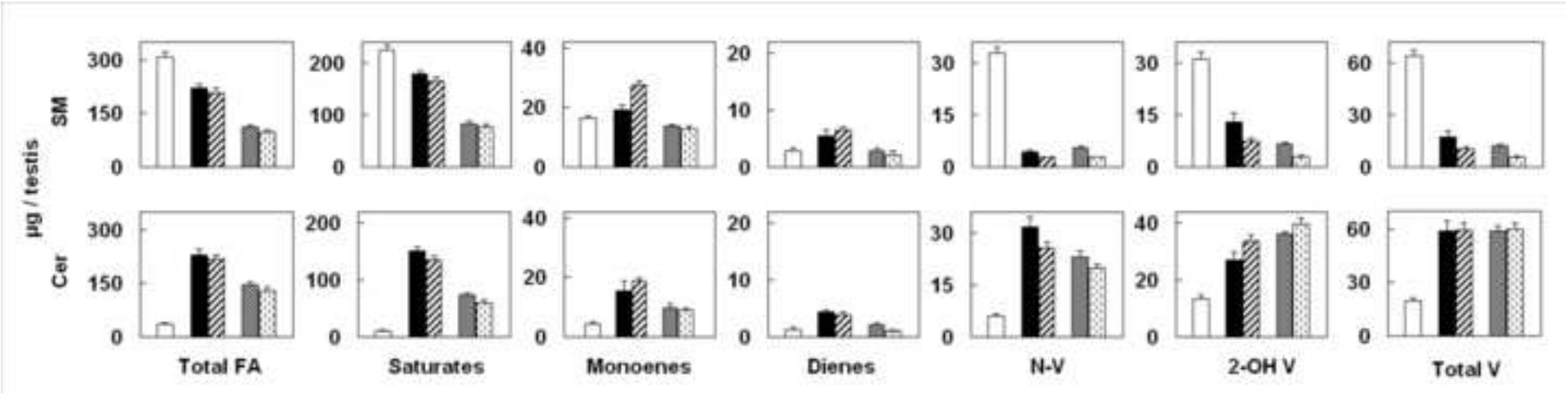

Scheme 1

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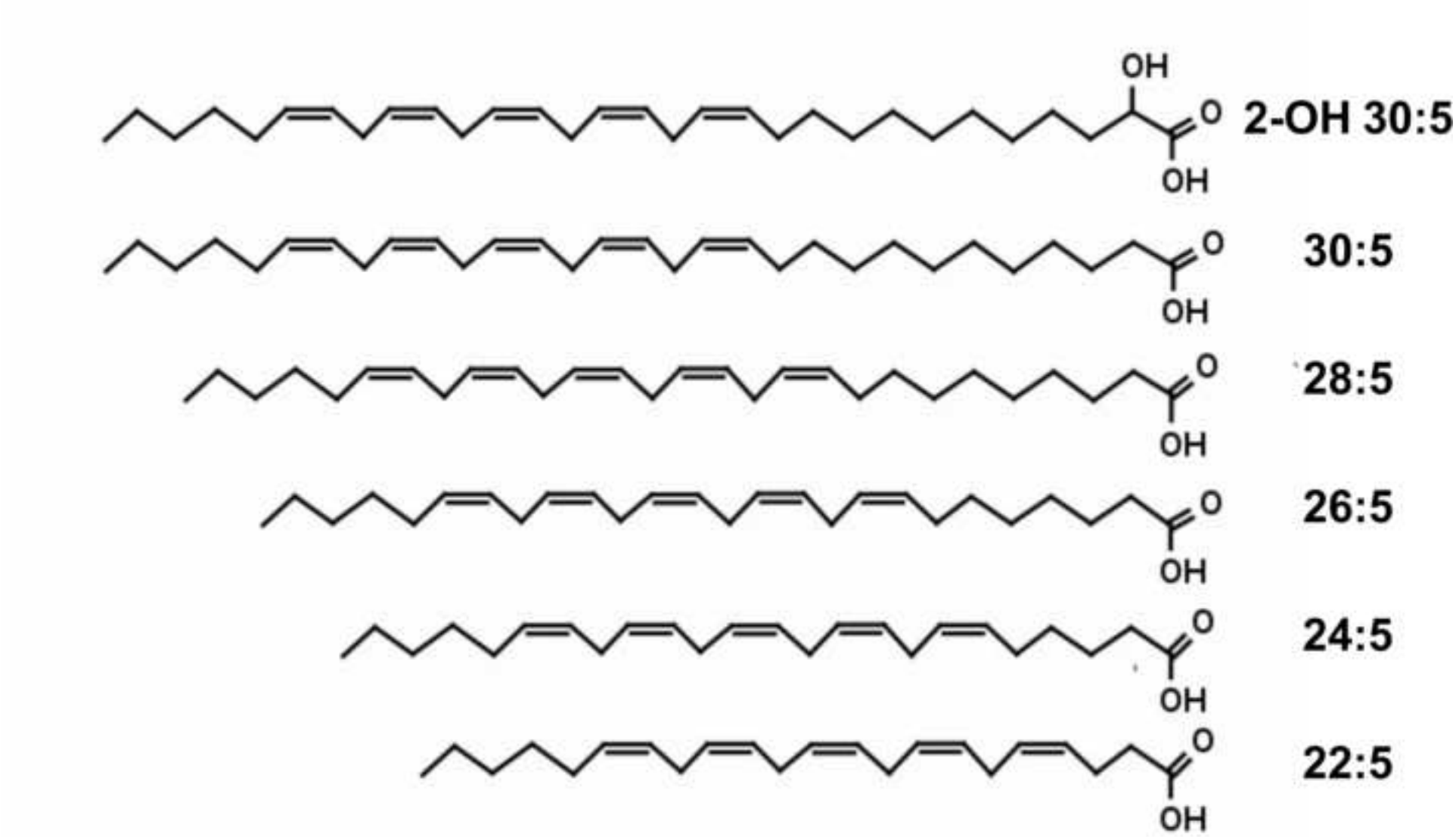

Scheme 2

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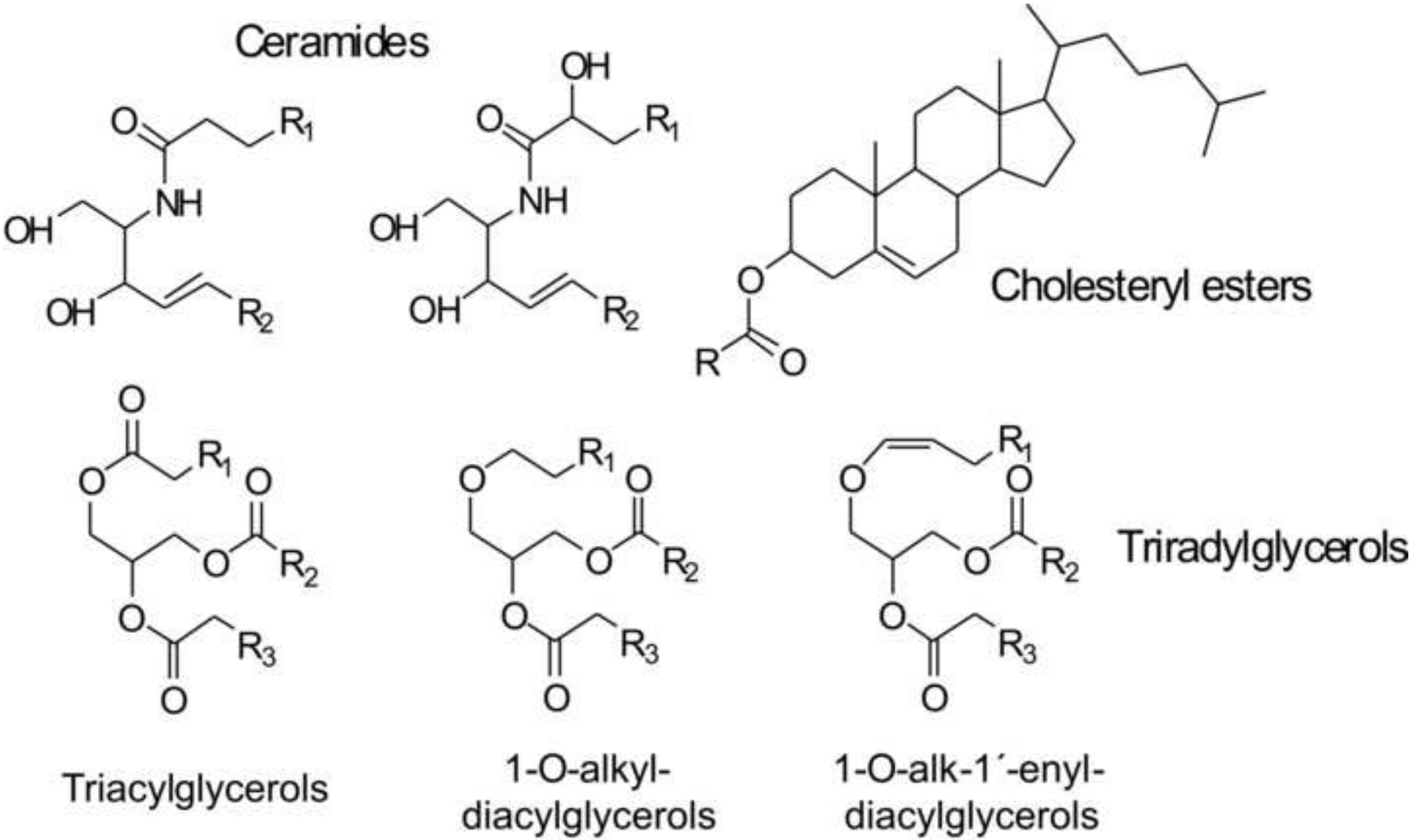

"Long-term biopermanence of ceramides, cholesteryl esters, and ether-linked triglycerides with very-long-chain polyunsaturated fatty acids in the cadmium-damaged testis"

Zanetti, S. R. and Aveldaño, M. I.

Supplementary figures

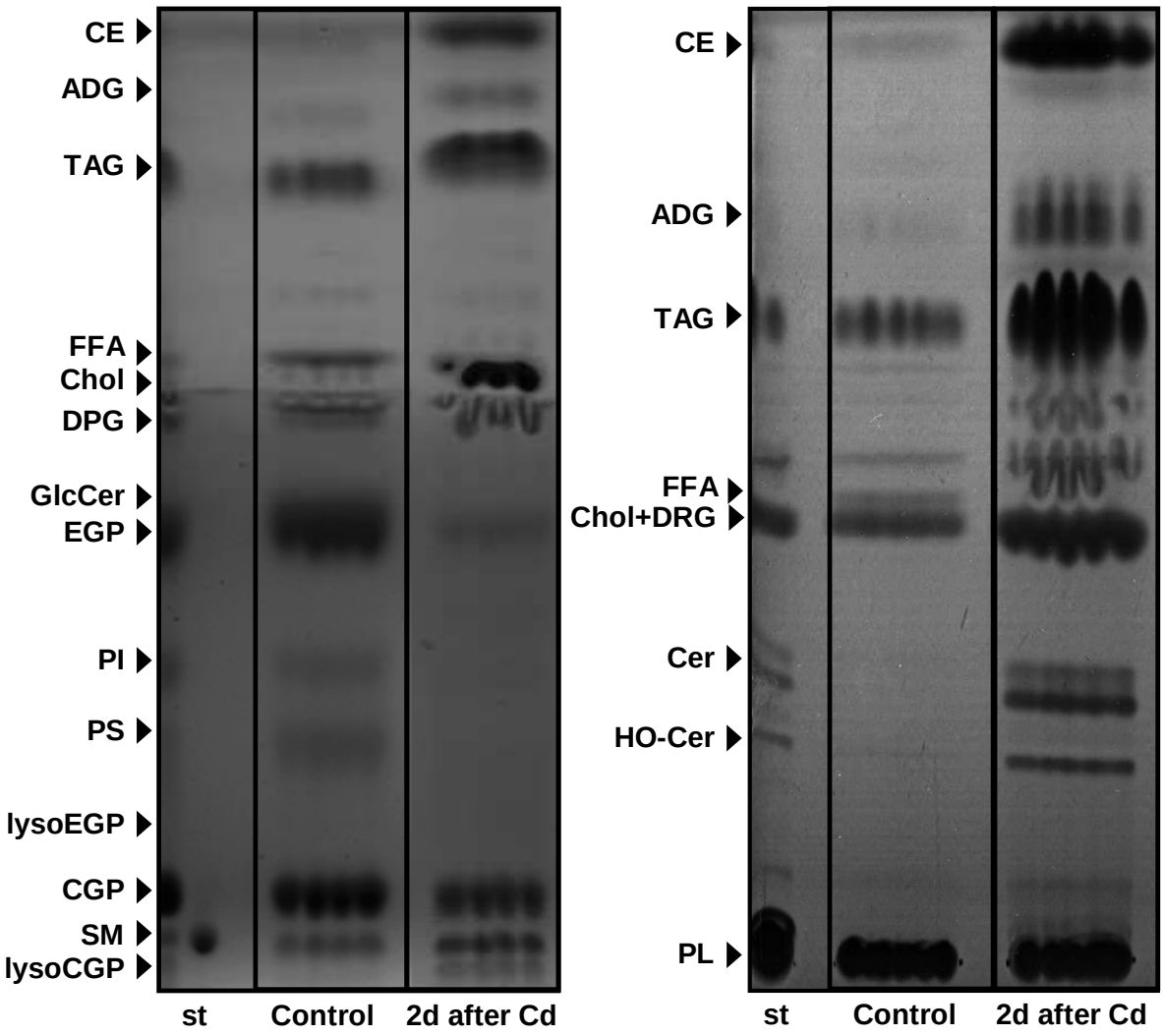

Fig. S1. TLC plates illustrating how Cd affects polar and neutral lipids of rat testis. In the plates on the left, chloroform:methanol: acetic acid:water (50:37.5:3.5:2, by vol) was run up to about 60% of the height of the plate to separate the phospholipids, after spotting aliquots equivalent so similar amounts of tissue weight. The plates were then dried and hexane:ethyl ether:acetic acid (80:20:1, by vol) was run up to the top to separate CE, ADG and TAG. On the right, a similar amount of lipid phosphorus (10 µg) was spotted and the plates were run up to about the middle of their height with hexane:ether:acetone:acetic acid (30:40:20:1, by vol) to separate ceramides, then dried and run with hexane:ether:acetic acid (80:20:2 by vol) up to the top to separate other neutral lipids. The plates were left for several hours in a tank with iodine vapors for visualization. Note on the right the band of ceramides with nonhydroxy fatty acids (here labeled Cer) separating into two bands: the upper one contains species with nonhydroxy VLCPUFA, and the lower one species with "shorter" fatty acids, such as 16:0). The Cer with 2-hydroxy- fatty acids (here labeled HO-Cer) forms a single band because it is made up exclusively of 2-hydroxy VLCPUFA amide-bound to sphingosine. GlcCer, glucosylceramide; PL, total polar lipids (mainly phospholipids); other abbreviations, as in the main text.

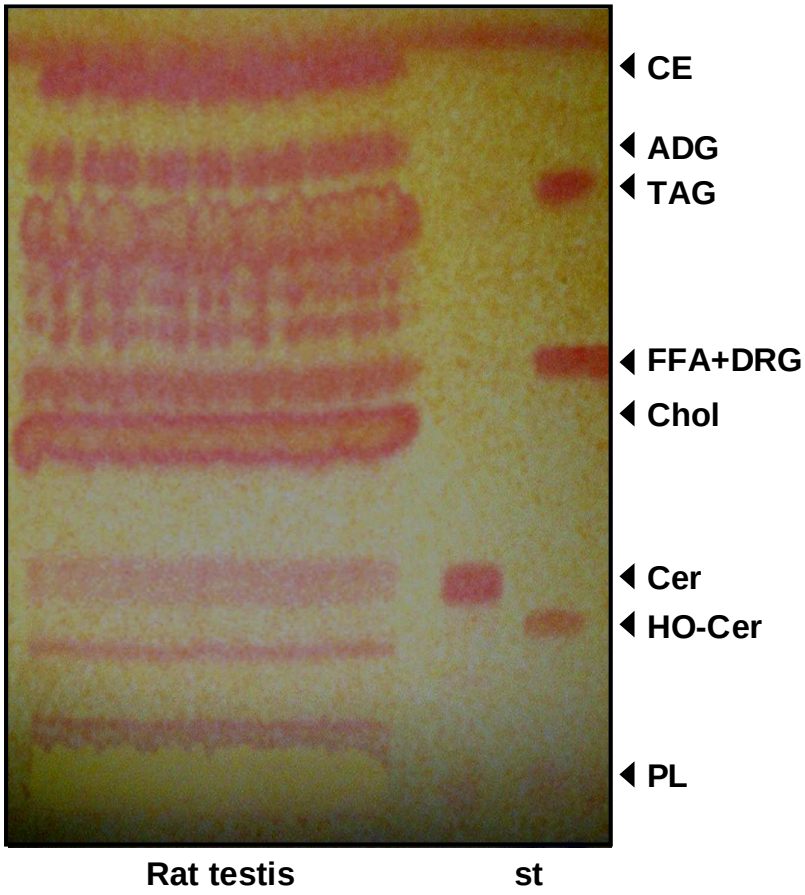

Fig. S2. TLC plate illustrating a preparative separation of neutral lipids from rat testis. 500 µg lipid P were spotted manually on a 500 µm thick silica gel G plate. The solvents described for the right panel of Fig. S1 were used for separation. The plate was then sprayed with 2,7´dichlorofluorescein in methanol (propelled by N_2) and observed under UV light. The different neutral lipids were eluted and either analyzed directly (e.g., ceramides) or subjected to further separations (e.g., ADG into subclasses as shown in Fig. S3).

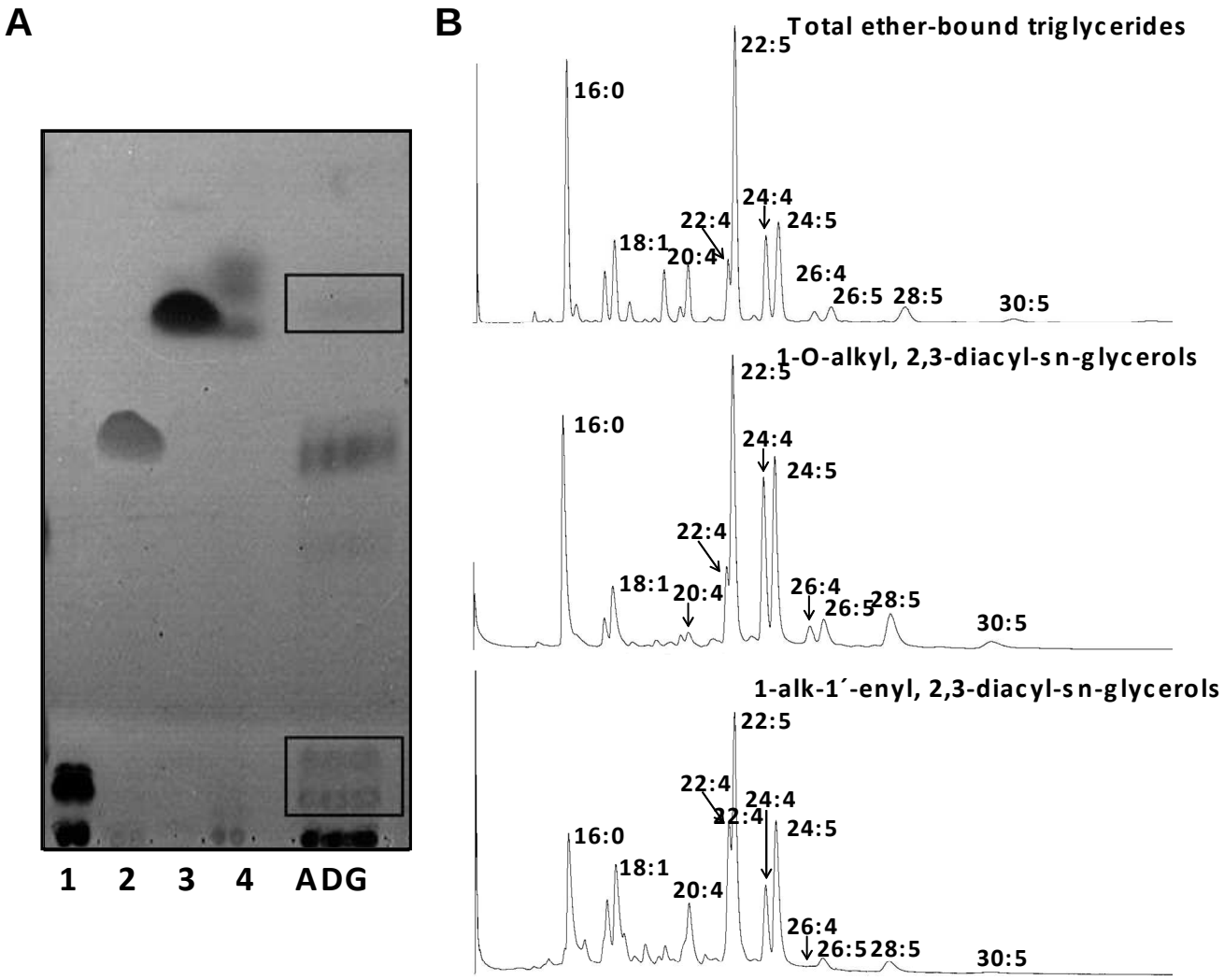

Fig. S3. **A.** Separation of ADG from the testis of an adult rat into 1-O-alkyl-*sn*-diacylglycerols (alkyl-DAG) and 1-alk-1´-enyl, 2,3-diacyl-*sn*-glycerols (alkenyl-DAG) subclasses. Total ADG, previously separated from TAG as illustrated in Fig. S2, were eluted, dried, and subjected to a brief (one minute) exposure to acid in chloroform-acetonitrile*, to take advantage of the lability of the 1-alk-1´-enyl bond to acid. This did not affect alkyl-DAG while producing a diacylglycerol and a fatty aldehyde (shown within rectangular frames) from alkenyl-DAG. The TLC on the right was run in two steps: first, hexane:ether (80:20, by vol) up to about half the height of the plate, then (after drying under N_2) hexane:ethyl ether (95:5, by vol) up to the top. 1, diacylglycerols; 2, 1-palmityl-2,3-dipalmitoyl diacylglycerol (alkyl-DAG); 3, palmitic aldehyde (commercial standards); 4, a small aliquot of fatty acid methyl esters (FAME) from testis total lipids; and ADG, an aliquot from rat testis ADG after being exposed to acid. *Note: Acetonitrile was used instead of methanol to avoid the formation of FAME from ester-bound fatty acids, as Rf of FAME and fatty aldehydes are close. Fatty aldehydes of rat alkenyl-DAG (not shown) were mainly 16:0, with smaller amounts of 18:0 and 18:1. **B.** Gas-liquid chromatographic profiles of the fatty acids of ADG before and after their separation into intact alkyl-DAG on the one hand and fatty aldehydes + diacylglycerols on the other (the fatty acids of the latter represent those of alkenyl-DAG). The latter are normally present in lower amounts than the former (see Fig. S4).

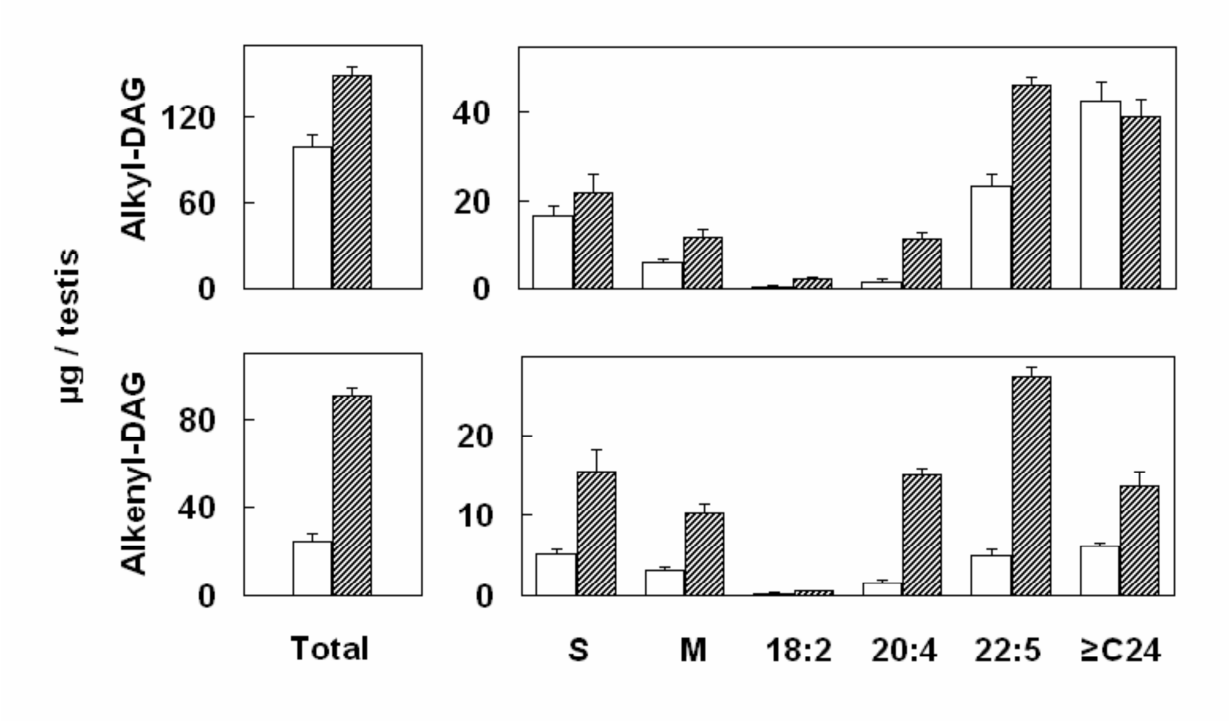

Fig. S4. Amount of the two subclasses of ADG, 1-O-alkyl, 2,3-diacyl-sn-glycerols (alkyl-DAG) and 1-alk,1´enyl, 2,3 diacyl-sn-glycerols (alkenyl-DAG), before and after one of the treatments with cadmium described in the manuscript. White bars: control animals; grey bars: 30 days after having started a regime of seven 1 mg/kg $CdCl_2$ doses, each separated from the other by 4 days. S, sum of saturated fatty acids; M, sum of monoenoic fatty acids; ≥C24, sum of very long chain (24 to 32 carbon atom) polyunsaturated fatty acids.

Long-term biopermanence of ceramides, cholesteryl esters, and ether-linked triglycerides with very-long-chain polyunsaturated fatty acids in the cadmium-damaged testis

Zanetti, S. R. and Aveldaño, M. I.

Supplementary Information

When the arterial blood supply to the testis is blocked transiently, spermatogenesis may be adversely affected even if the blood flow is subsequently restored. It resembles the normal response that follows the tissue damage that occurs after (rather than during) ischemia in many organs, especially the heart, classically known as ischemia-reperfusion injury. After 1 hour of testicular ischemia induced by a 720º testicular torsion followed by de-torsion and repair to allow for re-perfusion, Leydig and Sertoli cell functions are maintained, but a germ cell-specific apoptosis occurs which mainly involves spermatogonia in stages I and III of the seminiferous tubule epithelial cycle [3,7].

This apoptosis is detectable in rodent models as early as 4 hours and is maximal 24 hours after the repair of testicular torsion. Pro-inflammatory cytokines (e.g., TNFα, IL-1) are produced during testicular ischemia, which stimulates the expression, on the surface of endothelial cells, of adhesion molecules (e.g., E-selectin) that are responsible for the recruitment of leukocytes in subtunical venules of the testis during reperfusion [9]. These were identified as polymorphonuclear neutrophils (PMN).

Ischemia-reperfusion damage to the testis coincides with an increase in the level of PMN-derived reactive oxygen species (ROS). Germ cell apoptosis results from oxidative stress when the production of ROS during reperfusion *exceeds* the capacity of the antioxidant systems in the tissue. The finding that spermatogonia in active replication and differentiation to spermatocytes are the most affected by apoptosis after 1 hour of torsion ischemia followed by a few hours of reperfusion was attributed to the fact that they are the cells of the seminiferous tubules that are closer to the vessels [9].

In this section we describe the consequences of ischemia-reperfusion on testicular lipid classes, in order to contrast the progressive nature of the changes that affect lipids after this form of testicular injury with the devastating consequences of exposures to cadmium [11].

METHODS

Ischemia was induced in anesthetized animals after open surgery of the testis by a clockwise 720° torsion applied to the right spermatic cord. The right testis was maintained for 1 h in this torsion position by fixing it with a silk suture to the scrotal wall, followed by the return to the natural position to allow for reperfusion of the tissue [7] and scrotal suture. We gratefully acknowledge the invaluable skills as a surgeon of Dr. Eduardo N. Maldonado, who generously helped us by operating the animals. The testes were examined 1, 2, 7 and 30 days after torsion repair and healing. Lipid extracts were prepared from each of the testis separately, the left ones being used as controls. Testicular lipid classes were extracted and quantified on the basis of their fatty acids as explained for the cadmium-treated animals [11].

RESULTS and DISCUSSION

Ischemia-reperfusion

Two factors contribute to the gradual germ cell depletion that occurs as a consequence of the ischemia-reperfusion-induced damage to spermatogenesis. On the one hand, given the early irreversible disappearance of their precursor cells, spermatogonia, by apoptosis [9], new cohorts of germ cells stop to be formed. On the other, those more differentiated germ cells that were not lethally affected by ischemia-reperfusion progressively decreased in number mostly because they completed their differentiation, supported by Sertoli cells, and in due time abandoned the testis as spermatozoa towards the epididymis. This explains the fact that, one month after the ischemia-reperfusion episode, some of the tubules were populated only by Sertoli cells, whereas in others, Sertoli cells were still interacting with (the last) spermatids and spermatozoa (Fig. S1, left panels).

The tubules were smaller in diameter because they had fewer germ cells, but their lumena were patent, in clear contrast to the homogeneously-filled appearance of the tubules 30 days after cadmium treatments. The gradual reduction in germ cell number concurred with progressive decrease in testicular weight and in total lipid phosphorus content (Fig. S1, right panels).

Testicular glycerophospholipids (GPL), the major membrane lipid components of germ cells, decreased the most after the ischemia-reperfusion episode in the 1-30 day period that followed the ischemia-reperfusion episode (Fig. S2 A). The GPL species that contain 22:5n-6 (i.e., those of germ cells) contributed the most to this reduction, as observed from the fact that the percentage (%) of 22:5n-6 decreased more than that of other fatty acids in the decreasing mass of GPL (Fig S2 B).

Triacylglycerols (TAG), especially those rich in 22:5n-6, followed a similar trend of gradual decrease as did GPL (Fig. S2 A). This finds an explanation in the fact that 22:5n-6-rich TAG are formed in spermatids during the last stages of spermiogenesis, and are highly concentrated in the remnant particles known as "residual bodies" [5]. Thus, TAG decreased as the testes were depopulated of spermatids and spermatozoa, the last elements of the germ cell line to disappear from the testis.

Other two lipid classes whose amounts decreased gradually from the testis as a consequence of ischemia-reperfusion were sphingomyelin (SM) and ceramide (Cer) (Fig S3 A). In the 2-30 day post-ischemia-reperfusion period, the SM and Cer species that contain very long chain polyenoic fatty acids (VLCPUFA) contributed the most to the reduction in the amount of both lipids, as observed from the fact that the percentage (%) of this group of fatty acids decreased significantly within the decreasing mass of SM and Cer (Fig. S3 B).

In contrast to the situation after cadmium treatment, in this case *both* lipids decreased in parallel (no hydrolysis of SM), and the (non-hydroxy) VLCPUFA-containing species of both lipids virtually *disappeared* in 30 days. This is consistent with the previous observation that these species of SM and Cer belong to germ cells in meiosis, such as pachytene spermatocytes [5], since at day 30 these cells were no longer present. Regrettably, the species of SM and Cer with 2-OH VLCPUFA, which belong to more differentiated members of the germ cell line such as spermatids and spermatozoa [5], were not analyzed by GC after the present experimental conditions. However, as observed in our TLCs, although reduced, both were still present at this date, as previously observed 30 days after doxorubicin treatment [13] or 30 days after X-ray irradiation [4].

In contrast to the situation 30 days after Cd treatments [11], diglycerides, free fatty acids or ceramides did not accumulate after ischemia-reperfusion. The gradual decreases of 22:5n-6-rich species of GPL and TAG, and of the VLCPUFA-rich species of SM and Cer, is associated to completion of the last spermatogenic cycles and the egress of the formed spermatozoa from the testis. The fatty acids of these two membrane lipids reflect the fact that spermatogenesis continued for some time after the episode of ischemia-reperfusion at the expense of those germ cells that had escaped its deleterious effects.

In contrast to the decreases undergone by membrane-associated lipids, the amounts per testis of cholesterol esters (CE) and ether-linked triglycerides (ADG, 1-alkyl- and 1-alkenyl-diacylclycerols) increased from days 1 to 7 after ischemia-reperfusion (Fig. S2 A), and remained high at day 30. Taking into account the decreased testicular weight in that period (Fig S1) the concentration of both neutral lipids per gram of tissue augmented significantly. Because both lipids increased as germ cell numbers decreased, they most likely accumulated in Sertoli cells, as previously shown to occur after X-ray irradiation [4].

The PUFA rich species of CE that accumulated in testis after ischemia-reperfusion may have arisen in Sertoli cells after phagocytosis of apoptotic bodies derived from damaged germ cells, which are rich in GPL with 20:4n-6 and 22:5n-6 [5]. Free cholesterol and fatty acids derived from germ cell membranes may be combined in Sertoli cells and collected temporarily in lipid droplets, until cholesterol can be effluxed [6] and fatty acids oxidized [10].

In conclusion, ischemia-reperfusion resulted in gradually decreasing germ cell numbers with permanence of Sertoli cells, with essentially similar effects on lipids as previously observed after cryptorchidism [2], after treatments with the antineoplasic agent doxorubicin [12,13], after X-ray irradiation [4], and after mild hyperthermia [1]. It must be taken into account that all of these conditions, ischemia-reperfusion included, have in common that they initially induce testicular oxidative stress [8].

Ischemia with failed reperfusion

In a few of the rats of the present experiments, the described procedures failed to produce a successful reperfusion after testicular torsion. Regrettably, their testes showed, 24 hour later, the consequences of ischemia and associated necrosis. Although by accident, these bluish and edematous testes served i) as a negative control that in the rest of the animals the reperfusion procedures after ischemia had been successful,

and ii) as a positive control that most of the effects of 4 mg/kg $CdCl_2$ (manuscript, ref. [4]) at 48 h had occurred as a consequence of necrosis.

After 24 hours of ischemia (Table 1) the amount per testis of GPL decreased dramatically in the presence of increased amounts of diglycerides and FFA (Table S1). The latter two metabolites were rich in 20:4n-6 and 22:5n-6, just as the original GPL. Notably, TAG content and fatty acid composition were still similar to controls in that period. This suggests that testicular GPL breakdown due to necrosis precedes the accumulation of blood plasma- and cell- associated lipids including TAG that occurs in acute inflammation, as observed 48 h after Cd administration [11]. In both situations of ischemia followed by necrosis, the amount of total lipid per testis tended to decrease, suggesting that a part of the lipid-derived fatty acids was metabolized *in vivo*, oxidized and/or exported to the circulation, by cells that were not the ones that had died.

Most interestingly, a large proportion of the testicular SM was hydrolyzed to the corresponding ceramides after 24 h ischemia, resulting in a remarkable accumulation of Cer (Fig. S4). This resulted in a complete inversion of the Cer/SM mole ratio with respect to untreated animals.

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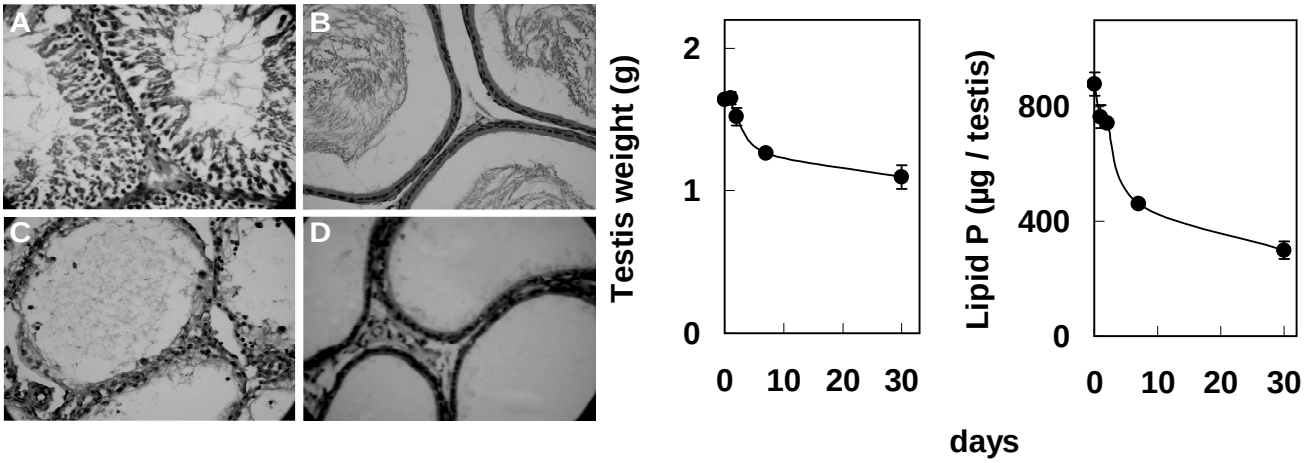

Fig. S1. Long-term effects of 1 hour of testicular ischemia followed by reperfusion in the rat testis. A and B, testis and epididymis, respectively, form a control rat; C and D, testis and epididymis, respectively, from a rat whose testis underwent 1 hour of ischemia followed by reperfusion, examined 30 days later. Note that 30 days after ischemia-reperfusion the tubules were smaller because they contained fewer germ cells than controls. The seminiferous tubules were populated mostly by Sertoli cells, some still containing (the last) spermatids and spermatozoa. Despite not all tubules were empty yet, their lumena were patent. The graphs on the right show the decrease in testicular weight and in lipid phosphorus.

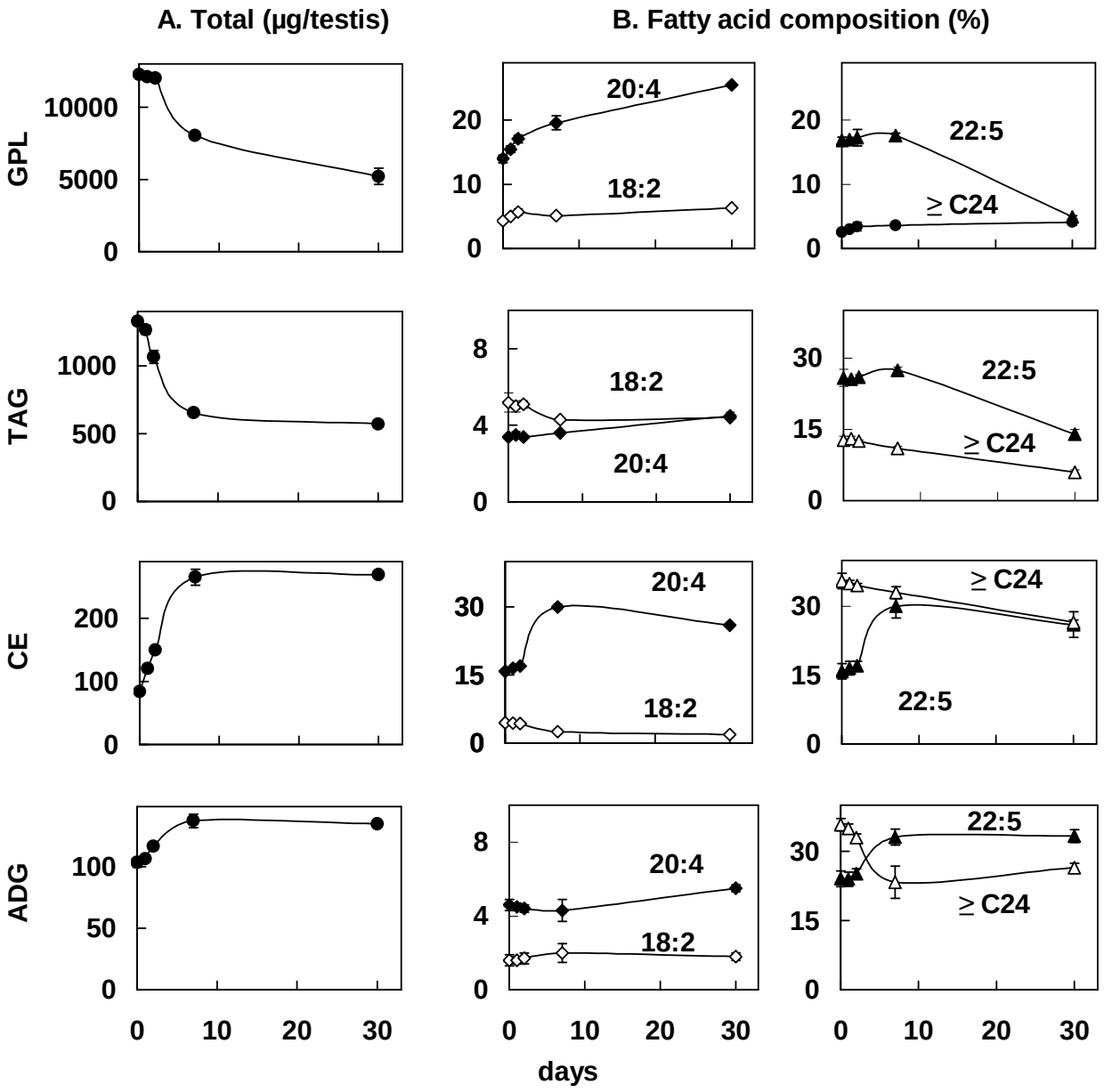

Fig. S2. Content (A) and percentage of main polyenoic fatty acids (B) of rat testicular glycerophospholipids (GPL), triacylglycerols (TAG), cholesterol esters (CE) and alkyl/alkenyl-diacylglycerols (ADG) as a function of time after an episode of 1 hour of ischemia followed by reperfusion and repair.

Fig S3

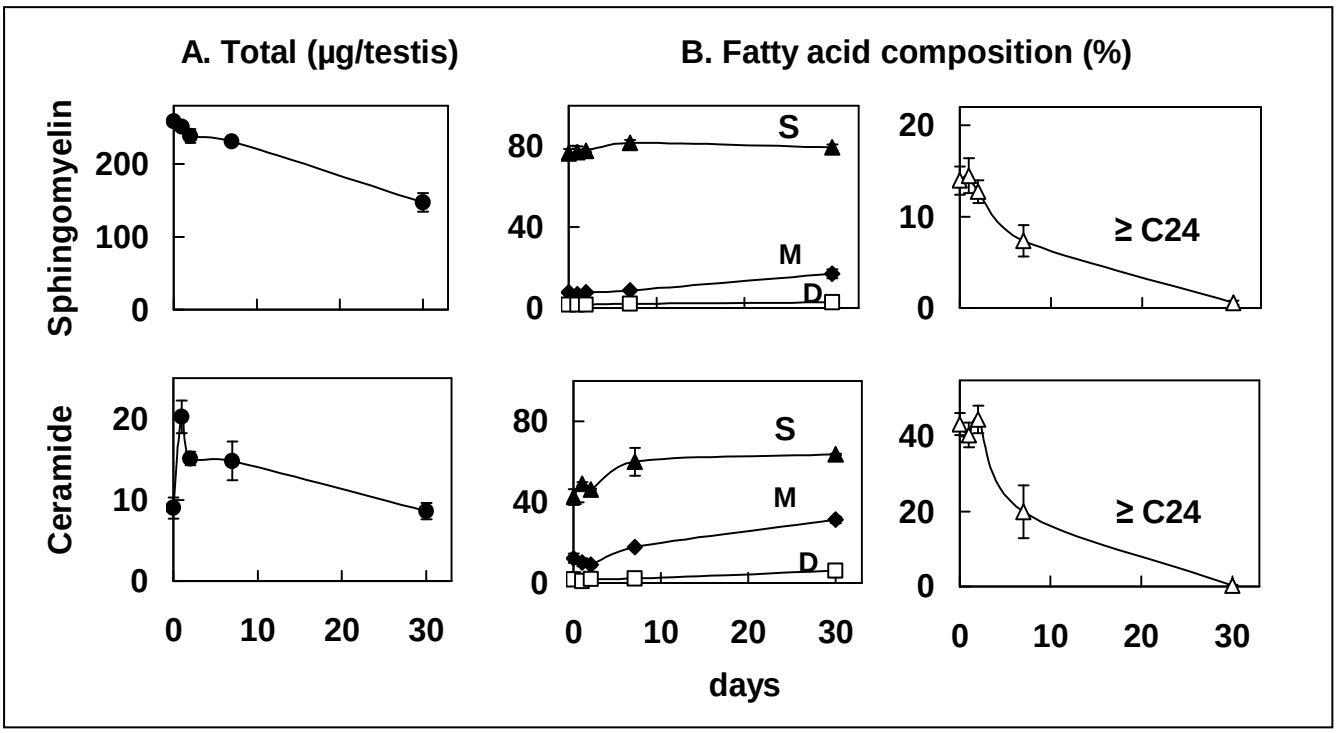

Fig. S3. Content (A) and percentage of main groups of fatty acids (B) of rat testicular SM and Cer as a function of time after an episode of 1 hour of ischemia followed by reperfusion. The species of both lipids marked as ≥C24 were nonydroxy VLCPUFA. These were the most decreased, disappearing from the testis in 30 days. The 2-OH VLCPUFA-containing SM and Cer were not analyzed by GC. Although smaller than in controls, they were still present, as shown 30 days after a single dose of doxorubicin [13] and 30 days after a single episode of X-ray irradiation [4].

Table S1. Lipid changes in rat testes undergoing ischemia with failure of reperfusion.

Lipid	Untreated	Ischemia
Total phospholipids	13310 ± 140	4105 ± 120
Free Fatty Acids	45 ± 3	502 ± 50
Diacylglycerols	93 ± 2	202 ± 5
Triacylglycerols	1480 ± 45	1451 ± 30
Ether-linked triglycerides	106 ± 3	109 ± 5
Cholesterol Esters	106 ± 4	212 ± 16

Data are given as µg/testis. After 1 hour of ischemia induced by testicular artery torsion, the anesthesized animals were treated as described to release the torsion and allow reperfusion of the testis. However, in the animals shown in this table reperfusion had failed and ischemia and its consequences were clearly apparent 24 hours after the operation, when they were sacrificed. The amounts of SM and Cer (µg/testis) in these conditions are shown in Fig S4.

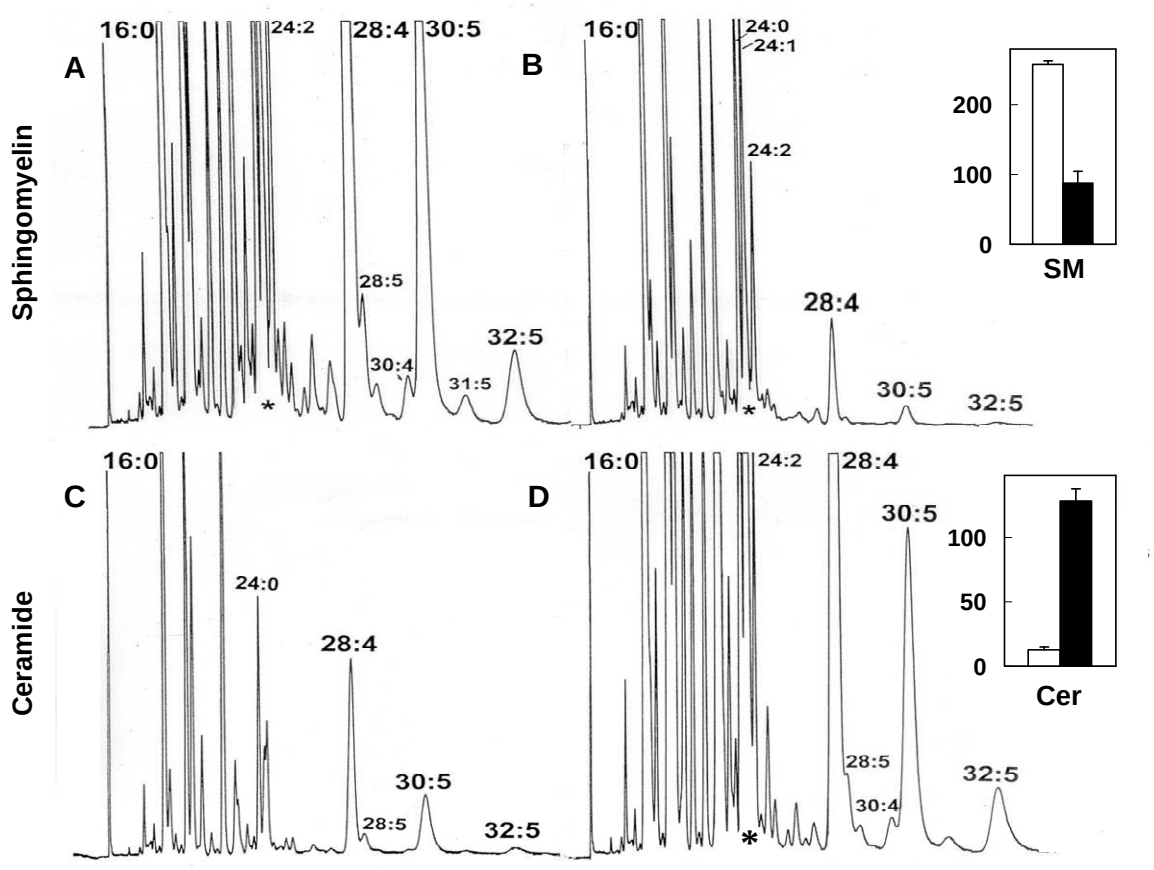

Fig. S4. Effect of 24 h ischemia on the amounts of rat testicular sphingomyelin (SM) and ceramide (Cer), as observed from their species with (nonhydroxy) fatty acids. A and B, SM from a control testis and a testis that underwent 24 h ischemia, respectively; C and D, the corresponding Cer from the same animals. Both lipids were isolated by TLC and their fatty acids analyzed by GC. The asterisks denote an increase in detector sensitivity. The same aliquots (1/10) of SM and Cer from a control and a damaged testis were injected to facilitate comparisons. In inserts, total amounts (µg/testis) of SM and Cer in control and ischemic testes (white and black bars, respectively). Amounts of other lipid classes are shown in Table S1.