BIOLOGY OF REPRODUCTION **85**, 128–136 (2011)
Published online before print 17 March 2011.
DOI 10.1095/biolreprod.111.091207

Mass Spectrometric Detection of Cholesterol Oxidation in Bovine Sperm[1]

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ABSTRACT

We report on the presence and formation of cholesterol oxidation products (oxysterols) in bovine sperm. Although cholesterol is the most abundant molecule in the membrane of mammalian cells and is easily oxidized, this is the first report on cholesterol oxidation in sperm membranes as investigated by state-of-the-art liquid chromatographic and mass spectrometric methods. First, oxysterols are already present in fresh semen samples, showing that lipid peroxidation is part of normal sperm physiology. After chromatographic separation (by high-performance liquid chromatography), the detected oxysterol species were identified with atmospheric pressure chemical ionization mass spectrometry in multiple-reaction-monitoring mode that enabled detection in a broad and linear concentration range (0.05–100 pmol for each oxysterol species detected). Second, exposure of living sperm cells to oxidative stress does not result in the same level and composition of oxysterol species compared with oxidative stress imposed on reconstituted vesicles from protein-free sperm lipid extracts. This suggests that living sperm cells protect themselves against elevated oxysterol formation. Third, sperm capacitation induces the formation of oxysterols, and these formed oxysterols are almost completely depleted from the sperm surface by albumin. Fourth, and most importantly, capacitation after freezing/thawing of sperm fails to induce both the formation of oxysterols and the subsequent albumin-dependent depletion of oxysterols from the sperm surface. The possible physiological relevance of capacitation-dependent oxysterol formation and depletion at the sperm surface as well as the omission of this after freezing/thawing semen is discussed.

cholesterol, cryopreservation, lipid peroxidation, mass spectrometry, oxidative stress, oxysterols, sperm, sperm capacitation

[1]Supported by the research program Biology of Reproductive Cells of the Graduate School of Animal Sciences from the Faculty of Veterinary Medicine of Utrecht University. P.F.N.S. is supported by Portuguese Foundation for Science and Technology, Ministry for Science, Technology, and Higher Education grant SFRH/2888/2000; R.A.v.G. was financed by ZonMw grant 903-44-156; A.B. was financed by the High Potentials program of Utrecht University; and N.G.-G. was funded by Spanish Ministry of Education and Science grant EX 2005-0460.
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Received: 21 January 2011.
First decision: 20 February 2011.
Accepted: 28 February 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

INTRODUCTION

Lipid peroxidation in sperm cells has received much attention during the past years, and beneficial as well as detrimental effects have been attributed to lipid peroxidation, resulting in the idea that pro-oxidant and antioxidant systems need to be carefully balanced for optimal sperm function [1–3]. On the one hand, mild reactive oxygen species (ROS) levels appear to be stimulatory for sperm capacitation [4, 5]. On the other hand, it is evident that extensive peroxidation damages sperm cells, because lipid radicals involved in propagation of the lipid peroxidation chain reaction (see Girotti [6] for an overview of the radical mechanisms involved) are rather indiscriminate toward the biomolecules they target. This extensive peroxidation will ultimately lead to functional impairment of proteins and DNA [7, 8]. One of the conditions that may induce extensive lipid peroxidation is the freeze/thaw process that is routinely performed in livestock breeding industries and human reproductive medicine. We previously confirmed that a freeze/thaw procedure induces peroxidation, and we were able to visualize this process in bovine sperm. In the same study we demonstrated the existence of peroxidized phospholipids [9]. However, the fate of the most abundant lipid molecule in sperm membranes, cholesterol, has remained unresolved.

Handling procedures for sperm storage can cause oxidation damage, as has been observed in food products. From studies on materials delivered from food industries it has been shown that oxidation of lipids is also of great importance and that cholesterol and its oxidation products are important diagnostic parameters for food quality because 1) cholesterol is abundantly present in lipid-rich foods of animal origin; 2) cholesterol is prone to auto-oxidation as well as radical-induced oxidation [10–12], thereby forming an excellent sensor of lipid peroxidation; and 3) several oxysterol species are toxic [13–17]. Although there are more than 30 different oxysterols known, only a few are quantitatively important [11, 18]. Oxysterols can be sensitively analyzed and quantified by liquid chromatography (LC) combined with atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) [13, 19]. Nevertheless, virtually no information is available on the formation of oxysterols in living cells.

The major aim of the present study is to determine the fate of cholesterol in sperm cell membranes. Cholesterol is the most abundant molecule in sperm cell membranes [20], and it stands to reason to apply the analysis of oxysterols to determine the extent of lipid peroxidation in sperm cells. Sterols have a function in sperm membrane stability and play an important role in sperm membrane organization and physiology [20–22]. On the one hand, cholesterol loading of sperm (mediated by cyclodextrins, for instance) improves their cryosurvival [23],

whereas depletion of cholesterol may turn sperm unstable and cause premature deterioration after artificial insemination or during incubations of in vitro fertilization [24]. On the other hand, a delicate regulation of cholesterol depletion by albumin has been reported to be instrumental for sperm capacitation in vitro [25, 26]. The present study focuses on the establishment of a mass spectrometric assay to detect the formation of oxysterols formed from cholesterol in sperm cells and the physiological significance of this process. Here, we show that sperm cells do indeed contain oxysterols, even directly after ejaculation. We report further about the effect of sperm handling procedures on additional oxysterol formation and on the fate of oxysterols during in vitro capacitation.

MATERIALS AND METHODS

Chemicals

All chemicals, including reference lipids, were obtained from Sigma Inc. (St. Louis, MO) and were of the highest purity available unless stated otherwise. Solvents (acetonitrile, chloroform, methanol, and hexane) were of high-performance liquid chromatography grade and were obtained from Labscan (Dublin, Ireland).

Sample Preparation

Bovine ejaculates were collected using an artificial vagina and were immediately processed to generate either fresh sperm samples or frozen/thawed sperm samples at a commercial artificial insemination center of Holland Genetics (Arnhem, The Netherlands). For fresh sperm samples, ejaculates were washed over a discontinuous Percoll gradient as described previously [27]. Pelleted cells were subsequently resuspended in Hepes-buffered Tyrode (HBT; 120 mM NaCl, 21.7 mM lactate, 20 mM Hepes, 5 mM glucose, 3.1 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, and 100 μg/ml kanamycin; 300 mOsm/kg, pH 7.4, supplemented with 0.5 mg/ml polyvinyl alcohol and 0.5 mg/ml polyvinylpyrrolidone), spun down at $750 \times g$ to remove any remaining Percoll, and were finally resuspended in HBT at a concentration of approximately 50 million cells per milliliter. These cells are further referred to as ''fresh cells.''

Frozen/thawed sperm samples were generated by resuspension of ejaculated cells in a freezing buffer and freezing in 0.25-ml straws in liquid nitrogen according to the method of van Wagtendonk-de Leeuw et al. [28]. After thawing for 30 sec at 38°C, cells were washed over a discontinuous Percoll gradient and with HBT as described above, and also resuspended at a concentration of 50 million cells per milliliter. These cells are further referred to as ''frozen/thawed cells.''

Artificial, protein-free membranes were made from extracted total sperm cell lipids as described previously [29, 30]. In brief, sperm lipids were extracted as described in the next section and were dried under nitrogen in a conical tube to form a lipid film. Lipids were hydrated with 96% (v/v) ethanol and dried again under nitrogen, and HBT was added before extensive vortexing. Finally, small unilamellar vesicles were formed by sonication on ice for 5×5 sec with the probe of a Soniprep 150 (MSE Scientific Instruments, Crawly, U.K.). Artificial protein-free lipid vesicles, fresh sperm, and frozen/thawed sperm were also incubated for 16 h in HBT either in absence of presence of 25 μM tert-butyl hydrogen peroxide to induce stress by radical oxygen species formation [31].

In Vitro Capacitation

Both fresh and frozen/thawed sperm samples were incubated for 4 h in presence of 0.5% (w/v) defatted bovine serum albumin (to replace polyvinyl pyrrolidone/polyvinyl alcohol) in HBT, or in polyvinyl alcohol/polyvinyl pyrrolidone containing HBT without albumin to induce sperm capacitation [20].

Extraction and Isolation of Oxysterols

The total lipid fraction from the sperm suspensions was extracted according to the method of Bligh and Dyer [32]. In addition, the sperm cells were spun down for 10 min at $10\,000 \times g$, and the supernatant was subjected to the same lipid extraction method to discriminate between the extracellular and the total oxysterol pools. Subsequently, cholesterol and oxysterols were separated from phospholipids by solid-phase extraction on 200-mg silica columns (Merck, Darmstadt, Germany). To this end, the lipid extract was dissolved in chloroform and applied to a column preconditioned with acetone, and the cholesterol plus oxysterol fraction was eluted with three volumes of acetone [19, 33]. Acetone was evaporated under a constant stream of nitrogen gas, and cholesterol and oxysterols were stored at −20°C until use.

High-Performance Liquid Chromatography-MS Analysis

Extracted (oxy)sterols were dissolved in a small volume of acetonitrile: methanol (6:4 [v/v]) and injected on an Aquasil C_{18} (250×4.6 mm, particle size of 5 μm) analytical column (Keystone Scientific, Bellefonte, PA) [13]. Elution was performed isocratically with acetonitrile:methanol (6:4 [v/v]) at a flow rate of 1 ml/min, and the column effluent was introduced by an atmospheric pressure chemical ionization (APCI) interface (Sciex, Toronto, ON) into a 4000 QTRAP mass spectrometer. For maximal sensitivity and for linearity of the response, the mass spectrometer was operated in multiple-reaction monitoring (MRM) mode at unit mass resolution. Peaks were identified by comparison of retention time and mass spectrum with authentic standards of the (oxy)sterols described (Merck). Ion transitions monitored were *m/z* 369.2/161.1 (cholesterol), 401.2/175.1 (7-ketocholesterol), and 367.2/159.1 (other oxysterols and desmosterol). When exploring the existence of other oxysterols (such as desmosterol-derived oxysterols) the mass spectrometer was operated in ''enhanced'' MS (ion-trapping) mode in the mass range 200–600 amu. Data were analyzed with Analyst software version 1.4.1 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

RESULTS

Detection, Identification, and Quantification of Oxysterols

(Oxy)sterols can be ionized readily by APCI (Fig. 1A). In accordance with previous reports [13, 32], (oxy)sterols were not observed as (quasi)molecular ions, but had lost any hydroxyl groups (including that on C-3) as a molecule of water (see Fig. 1A, inset). Product spectra of the $[M+H-H_2O]^+$ ions generated by collisional activation were complex, and structural information was hard to obtain from these spectra because they consisted of numerous fragment ions that were common to product spectra of many (oxy)sterols (Fig. 1, B–D). The discrimination between (oxy)sterols was therefore based on the different retention times of the oxysterols during reverse-phase chromatography as detected by evaporative light scattering detection (Fig. 2A) or mass spectrometry (Fig. 2B). Calibration curves for quantification purposes were constructed in MS/MRM mode for eight (oxy)sterols that are of physiological importance. All calibration curves were linear up to at least 100 pmol and had correlation coefficients (r^2) between 0.99 and 1.00. The (oxy)sterols were detected with small variations in sensitivity due to differences in their ionization and fragmentation efficiencies, but had limits of detection of around 50 fmol (oxysterols) to 200 fmol (cholesterol and desmosterol; Fig. 3). For assessment of lipid peroxidation, the absolute amounts of cholesterol, desmosterol, and individual oxysterols were calculated using the corresponding calibration curves. Oxysterols were expressed as mole-% of cholesterol.

Oxysterols Are Present in Fresh and Frozen/Thawed Sperm Cells

Analysis of Percoll-washed, fresh sperm cells, from which lipids were extracted as soon as feasible after ejaculation, revealed the presence of cholesterol, desmosterol, and oxysterols in these cells (Fig. 4A). All oxysterol levels were highly increased (approximately by a factor of 6) when cells were stressed with tert-butyl hydrogen peroxide, whereas their relative contribution to the total amount of oxysterol resembled that of unstressed cells (Fig. 4B). However, in protein-free reconstituted vesicles from sperm lipid extracts the production of oxysterols under the same tert-butyl hydrogen peroxide

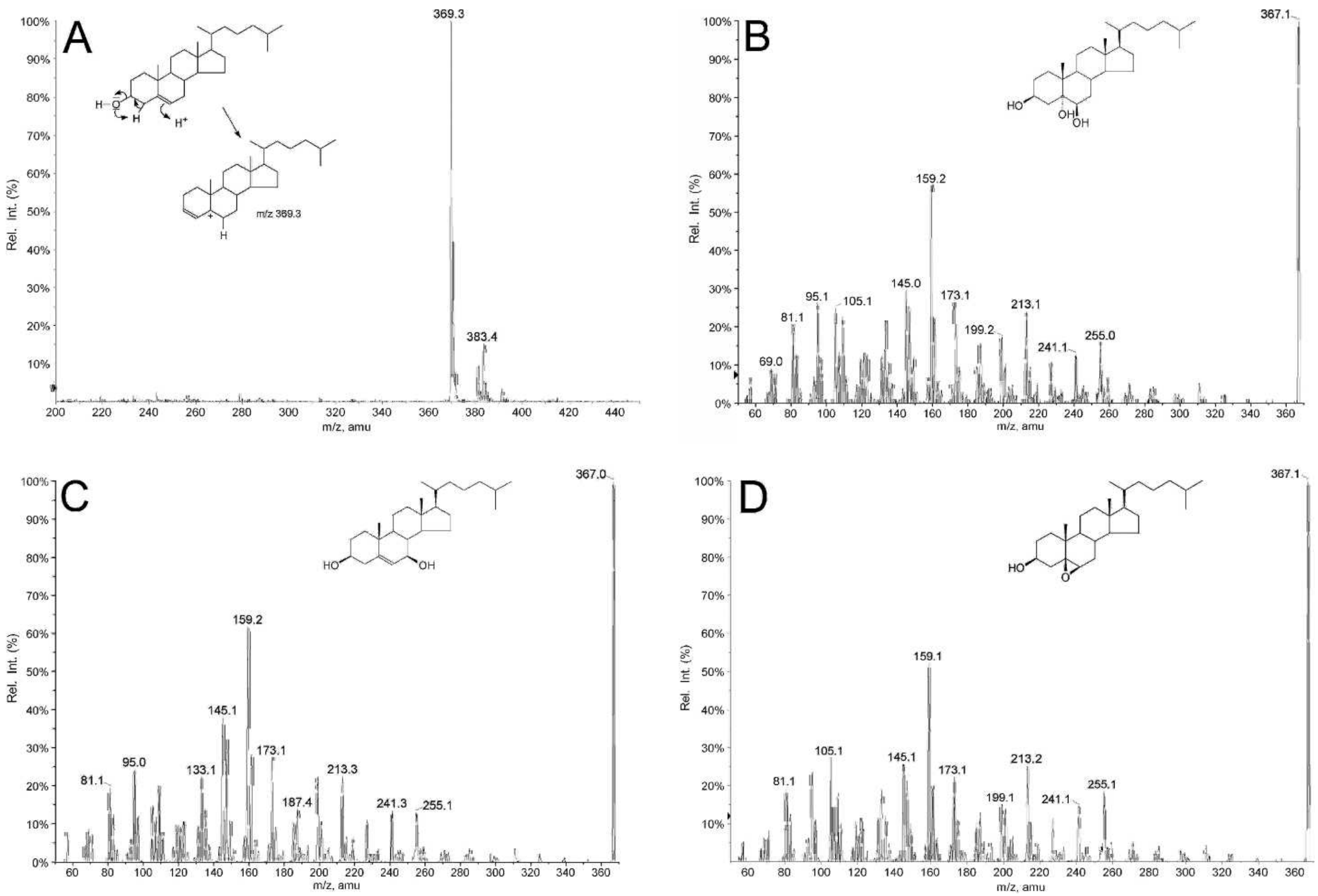

FIG. 1. Mass spectrometry of authentic standards of (oxy)sterols. **A**) Mass spectrum recorded during the elution of cholesterol. The inset shows a proposed mechanism for the formation of the $[M+H-H_2O]^+$ base peak ion during APCI ionization. **B**–**D**) Indiscriminate and extensive fragmentation of (oxy)sterols. Fragmentation spectra of (oxy)sterols at 35-V collision energy. **B**) Cholestane-3,5,6-triol. **C**) 7β-Hydroxycholesterol. **D**) 5,6β-Epoxycholesterol. Rel. Int., relative intensity.

stress doubled (Fig. 4C), which may imply either that the sperm cells possess an antioxidant network preventing cholesterol oxidation or that a higher proportion of cholesterol in sperm cells was not exposed to tert-butyl hydrogen peroxide compared with the reconstituted vesicles. The peroxidation of protein-free vesicles also led to the formation of different oxysterols, most notably to the reduced abundance of 7-ketocholesterol and the more abundant presence of cholestane-3,5,6-triol. One additional oxysterol was observed that was not detected in living sperm cells (Fig. 4C, asterisk). Based on its retention time and its fragmentation spectra in positive and negative modes, it was tentatively identified as 7-hydroperoxycholesterol (data not shown). Note that higher sterol oxidation led to lower cholesterol:desmosterol ratios (Fig. 4).

In addition, based on the identified oxysterol products, indeed apparently cholesterol is predominantly attacked by ROS at three places (illustrated in Fig. 5), of which the attack to the 25 carbon atom of cholesterol is minimal. Reaction products indirectly created after the radical oxygen species collision at the 7 or the 5 and 6 carbon atoms of cholesterol are indicated with arrows in Figure 5.

When sperm cells underwent the freezing protocol (taking a total time of approximately 3 h) and were subsequently thawed, the total amount and composition of oxysterols in these cells remained unaltered compared with fresh cells, showing that the freezing procedure itself did not result in elevated oxysterol levels ($P = 0.52$, n = 3; Fig. 6). Subsequent incubation of fresh or frozen/thawed sperm cells for 4 h did not change the oxysterol pattern in frozen/thawed sperm ($P = 0.12$, n = 3). In contrast, the amount of oxysterols increased when fresh sperm were incubated in HBT for 16 h ($P = 0.03$, n = 3; Fig. 6). The composition of the cholesterol oxidation products was similar for all sperm incubations but differed from the reactive oxygen-stressed protein-free vesicles made from reconstituted sperm lipid extracts (Fig. 6).

Fresh and frozen/thawed sperm cells were incubated for 4 h in HBT without or with 0.5% albumin (in vitro capacitation) to determine whether albumin has an affinity for the oxysterols. Clearly, as depicted in Table 1, only fresh sperm showed a significant incubation-dependent formation of oxysterols, and the vast majority of these oxysterols were extracted in the presence of albumin. Both responses were found to not be significant in frozen/thawed sperm during the same incubations.

DISCUSSION

Sperm cells contain high amounts of polyunsaturated phospholipids, which makes them particularly vulnerable to lipid peroxidation [33, 34]. Many papers have demonstrated

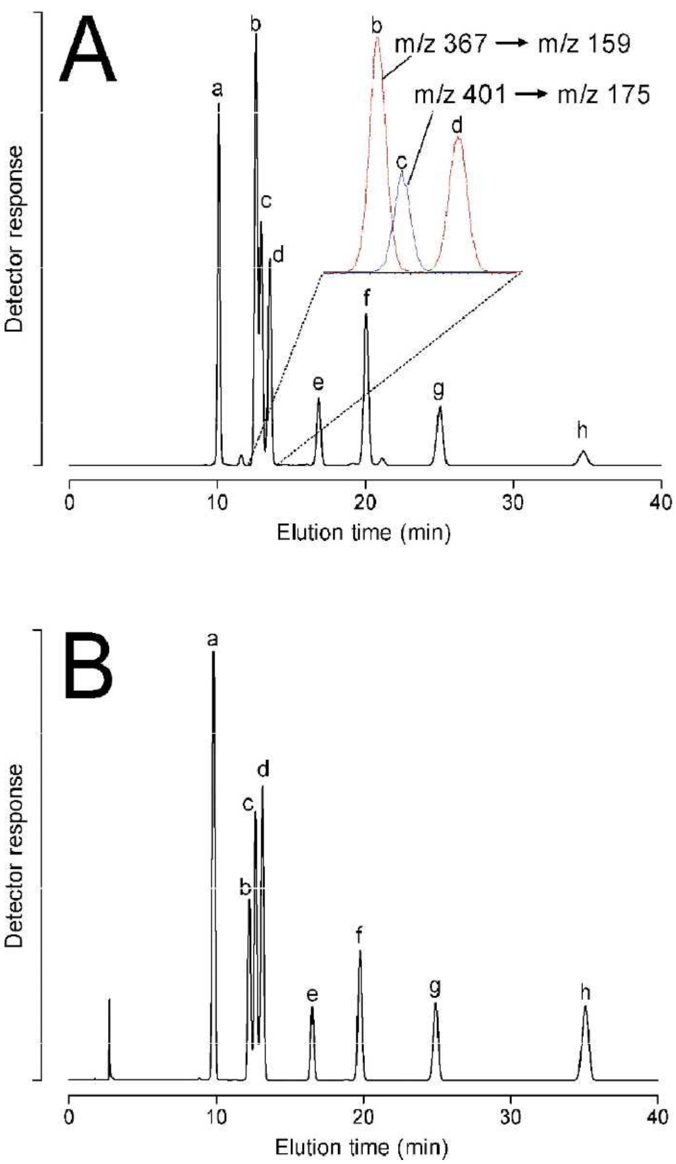

FIG. 2. Detection of (oxy)sterol standards by MRM-MS (**A**) or evaporative light scattering detection (**B**). Standards shown are: cholestane-3,5,6-triol (**a**); 7β-hydroxycholesterol (**b**); 7-ketocholesterol (**c**); 25-hydroxycholesterol (**d**); 5,6β-epoxycholesterol (**e**); 5,6α-epoxycholesterol (**f**); desmosterol (**g**); and cholesterol (**h**). The inset in **A** shows distinction between oxysterols based on different mass spectrometric fragmentations.

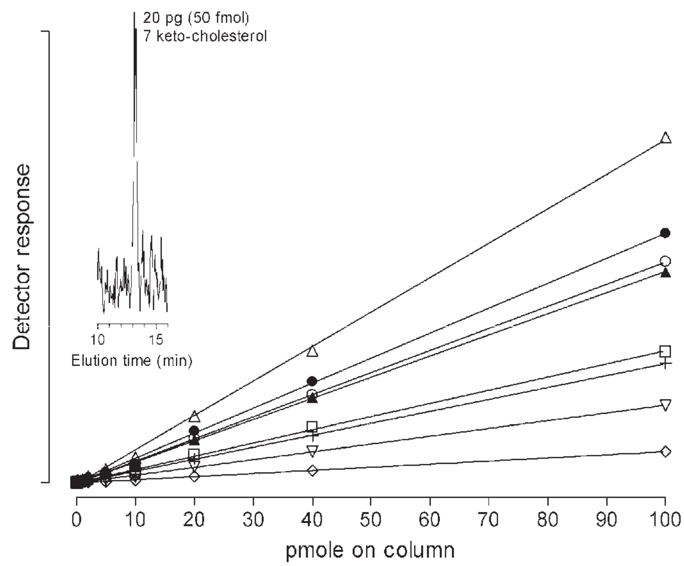

FIG. 3. (Oxy)sterols produce linear calibration curves over a broad dynamic range. Differences in sensitivity result from differences in ionization and fragmentation efficiencies. From top to bottom: 7β-hydroxycholesterol; cholestane-3,5,6-triol; 5,6α-epoxycholesterol; 25-hydroxycholesterol; 7-ketocholesterol; desmosterol; and 5,6β-epoxycholesterol and cholesterol. The inset shows the signal:noise ratio at the lower level of detection of 7-ketocholesterol.

the occurrence of lipid peroxidation during incubation of sperm cells under various conditions [2, 9, 34–36]. However, because these experiments have typically assessed the formation of such lipid peroxidation products as malondialdehyde and 9-hydroxynonenal during incubation, little is known about the amount of other lipid peroxidation products (e.g., oxysterols) in sperm cells directly after ejaculation. Additionally, it is important to realize that in the female genital tract, sperm cells are exposed to an exogenous peroxidative environment, and that this environment is both believed to support sperm activation (under mild radical oxygen species conditions [4, 5]) or may be detrimental to the sperm (for instance, when sperm and leukocytes are in contact and extensive radical oxygen species formation takes place). It is therefore of importance to analyze both the sperm cell's ''load'' of oxysterols directly after ejaculation and after sperm processing for cryopreservation or during in vitro fertilization treatments. The rationale to set up the oxysterol detection in sperm was 2-fold, namely, 1) cholesterol itself is believed to stabilize sperm, which may attribute to its suitability for successful freeze/thaw procedure survival [23], and 2) on the other hand, sperm capacitation is known to depend on albumin-mediated cholesterol depletion, and an unclear link is reported regarding mild formation of ROS and sperm capacitation as well [4, 5, 37].

Mass Spectrometry

Our observed APCI mass spectra of the individual oxysterols were identical to the spectra shown for processed foods by Razzazi-Fazeli et al. [13]. Many oxysterols are observed at identical mass:charge ratios, particularly after the loss of hydroxyl groups as molecules of water, a typical event during the APCI ionization process. Therefore, it was investigated whether discrimination between oxysterols could be made on basis of their fragmentation spectra. This would allow the oxysterol-specific fragments to be used for mass spectrometric multiple-reaction monitoring during liquid chromatography, which is a sensitive and selective technique for the detection of components. The resulting fragmentation spectra after collisional activation of cholesterol and oxysterols showed particular extensive and indiscriminate fragmentation. This clearly implicates that it is not possible to discriminate between (oxy)sterols based solely on their fragmentation spectra, and that chromatography is an indispensable tool to first separate the different types of (oxy)sterols, allowing their mass spectrometric identification. Moreover, many fragment ions showed similar intensities, meaning that there was no fragmentation pathway yielding high amounts of a particular ion to be used in multiple-reaction monitoring. Despite the lack of specific, high-abundance fragment ions, a detection limit of approximately 50 fmol of oxysterol and a linear dynamic range of more than three orders of magnitude were obtained. Approximately 0.1% of cholesterol has been converted into a

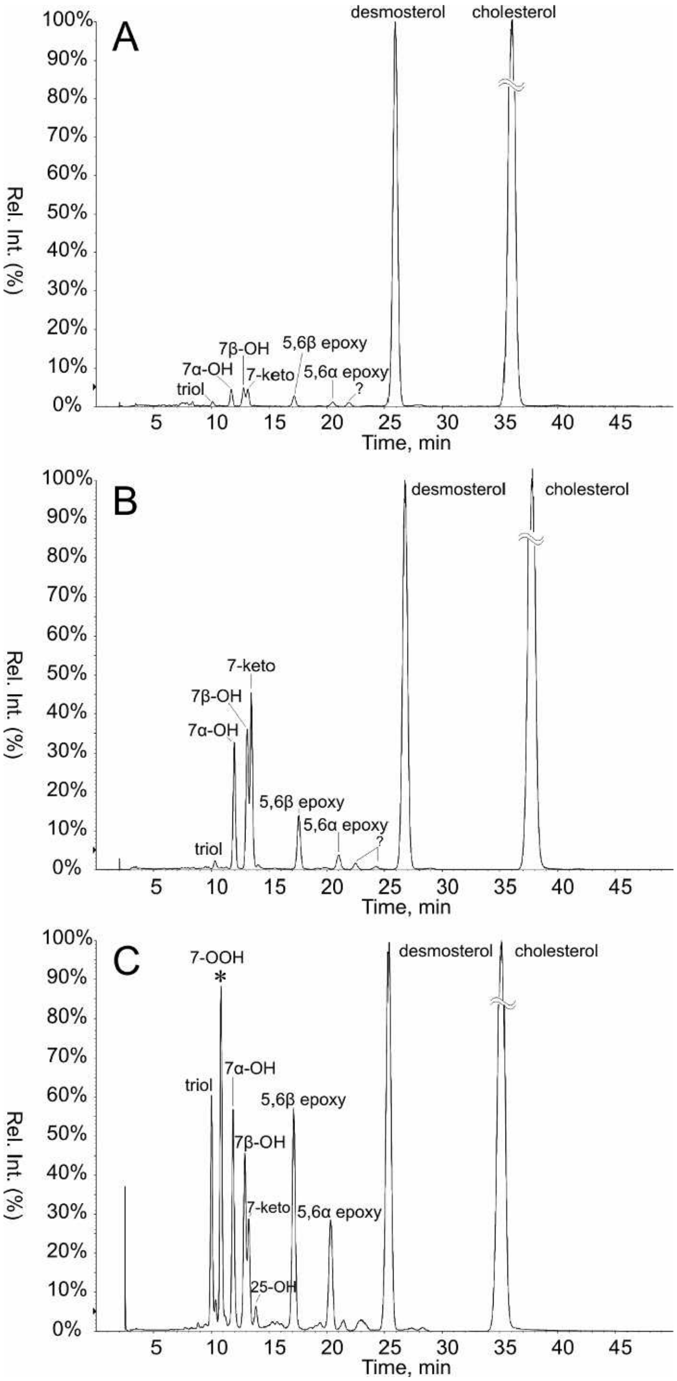

FIG. 4. Detection of (oxy)sterols in Percoll-washed sperm cells by MRM-MS. **A**) Bovine sperm cells directly after ejaculation. **B**) Sperm cells incubated in the presence of oxidants. **C**) Small unilamellar vesicles constructed from a sperm cell lipid extract incubated in the same presence of oxidants as in **B**. Figures are normalized to desmosterol for clarity. Compared with the normalized levels of desmosterol, the cholesterol peaks in the chromatograms of **A**, **B**, and **C** were 679%, 622%, and 578%, respectively. Rel. Int., relative intensity.

particular oxysterol (see above). From this number one can calculate that the amount of one oxysterol species can be detected in 50 000 sperm cells. This illustrates that commercial cell sorters (able to sort cells at typical rates of up to 10 000 cells per second) can easily provide us with sorted sperm subpopulations for investigation of their lipid peroxidation profiles under variable physiological conditions.

TABLE 1. Formation and albumin-dependent extraction of oxysterols in fresh and frozen/thawed bull sperm before and after in vitro capacitation.[a]

Sperm sample	0 h	4 h (− albumin)	4 h (+ albumin)
Fresh semen			
Oxysterol/cholesterol (%)[b]	0.39 ± 0.12	1.43 ± 0.24[d]	1.72 ± 0.25[d]
Oxysterol in sperm (%)[c]	98 ± 2	95 ± 3	14 ± 3[d]
Frozen/thawed semen			
Oxysterol/cholesterol (%)[b]	0.41 ± 0.09	0.56 ± 0.18	0.57 ± 0.27
Oxysterol in sperm (%)[c]	94 ± 3	90 ± 4	83 ± 8

[a] Mean values ± SD are provided; data are obtained from seven bulls, using three individual ejaculates (n = 7, r = 3).
[b] The relative amount of total oxysterol formed from cholesterol (100%) is indicated.
[c] The relative amount of the formed oxysterols (100% in semen) which was recovered in the sperm cell pellet after centrifugation, and the remaining oxysterols were recovered in the supernatant.
[d] Data differed significantly from the 0 h condition.

Presence of Oxysterols in Fresh Sperm Cells

Oxysterols are still very hydrophobic molecules that are retained in the cellular membranes. Combined with the low level of detection of oxysterols (typically around 50 fmol), this makes analysis of oxysterols the technique par excellence for the assessment of the total oxidative stress cells have encountered. In contrast, peroxidation of phospholipids results in the formation of hydrophilic end products such as malondialdehyde and 9-hydroxynonenal, components that are water soluble and therefore do not remain associated with the sperm cell. Assessment of lipid peroxidation by fluorescent techniques has also successfully been performed in sperm cells, but this only detects oxidative stress from the moment cells have been fluorescently labeled [9, 35], and not (as is the case with oxysterols) the oxidative stress that a cell has encountered so far. Therefore, in our view the detection of oxysterol levels in ejaculated semen samples can be used as a diagnostic tool for screening sperm quality because too high levels of oxysterols reflect too high oxidative stress, which is well known to impair sperm functioning [37].

Analysis of sterols in sperm cells directly after ejaculation revealed the abundant presence of cholesterol and desmosterol, as expected from previous data [38–40]. Small amounts of oxysterols were also clearly present in fresh and in frozen/thawed sperm, demonstrating that sperm cells have been subjected to peroxidation prior to the time they were ejaculated. Based on their retention time and their molecular weights, the observed oxysterols appeared to be derived from cholesterol and not from desmosterol. This is remarkable, considering the fact that around 16% of the sterol in bovine sperm cells is desmosterol, and that the only chemical difference between cholesterol and desmosterol is the double bond between carbons 24 and 25 in desmosterol. Theoretically, desmosterol would be expected to be a better substrate for peroxidation because double carbon atom bonds are preferred sites of oxidation. 25-Hydroxycholesterol is the only oxysterol that could partly be derived from desmosterol, because the double bond between carbon atoms 24 and 25 in desmosterol would have been lost during oxidation. The fact, however, that 25-hydroxycholesterol was only a minor oxidation product in our experiments points toward a different underlying reason for the observed preferred oxidation of cholesterol. One might consider the possibility that the 25 carbon atom of both cholesterol and desmosterol is localized in the membranes in such a way that it is almost hidden for ROS. We are currently performing oxidation assays on sterols in homogeneous solution as well as studies on artificial membranes with various

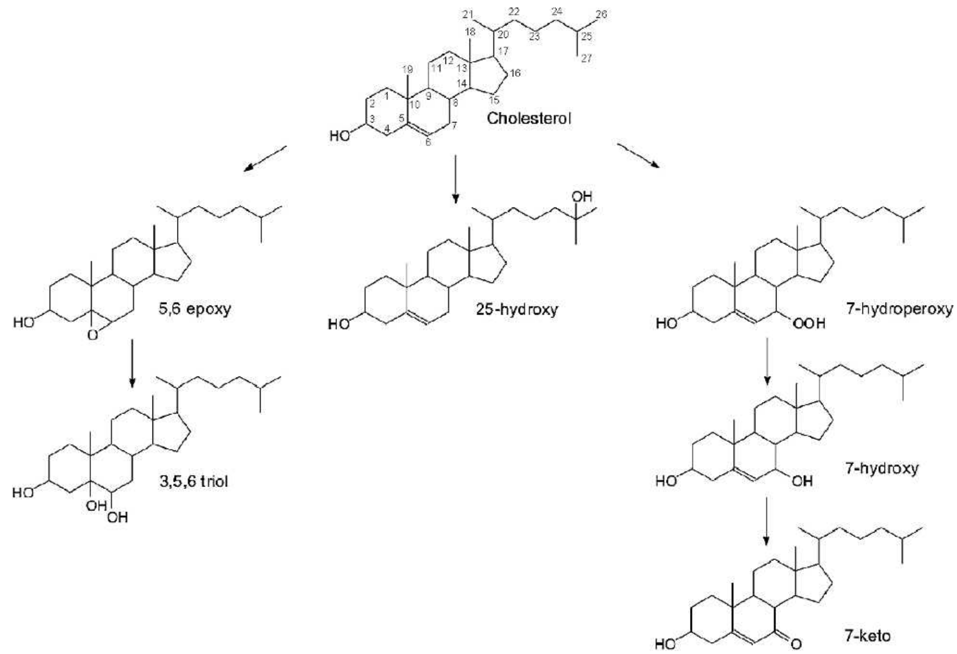

FIG. 5. Molecular interpretation of cholesterol oxidation pathways identified in bovine sperm.

lipid compositions to elucidate this distinct behavior of cholesterol and desmosterol.

Inhibition of Oxysterol Formation by Sperm Cells

To obtain insight into whether or not sperm cells have the capacity to inhibit oxysterol formation, we compared this process under imposed reactive oxygen stress in both sperm cells and artificial vesicles reconstituted from sperm cell lipid extracts. Striking differences in the amount and composition of oxysterols were found between these samples, although the oxidative stress was generated in an identical fashion. This suggests that cholesterol in sperm cells is protected for oxysterol formation (probably by means of an active radical scavenging

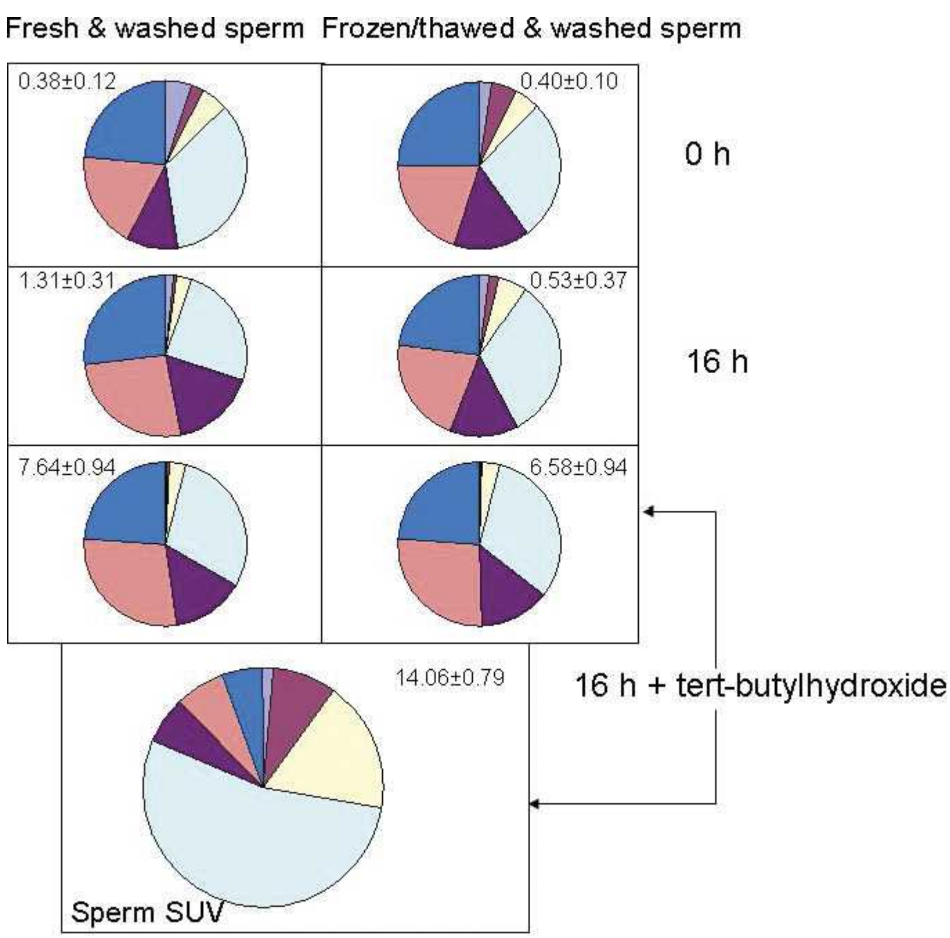

FIG. 6. Composition of oxysterols in sperm treated as indicated and in reconstituted small unilamellar vesicles from extracted sperm cell membrane (sperm SUV). Mean ± SD values of total oxysterol formation (in % of total cholesterol) are provided (n = 3). Oxysterol species are presented in following order starting from the north and continuing clockwise: 25-hydroxy (light purple); 3,5,6-triol (dark brown); 5,6α-epoxy (yellow); 5,6β-epoxy (light blue); 7α-hydroxy (dark purple); 7-keto (light brown); and 7β-hydroxy (dark blue). For structures, see Figure 5.

system). From the apparent differences in specific oxysterol species, one may hypothesize that sperm cells metabolize oxysterols. However, we loaded sperm uniform ^{2}H isotope-labeled oxysterols (prepared from ^{2}H cholesterol; each of the labeled forms of oxysterols as depicted in Fig. 5 were made) and found that bovine sperm did not convert any of these ^{2}H oxysterols at all (data not shown). The best explanation between the different oxysterol species found in the reconstituted vesicles compared with sperm is that cholesterol orientation in the vesicles is different from cholesterol in the parent physiological membranes of the living sperm bilayer from which they were extracted. For instance, lipids are asymmetrically oriented in sperm membranes [41, 42], and cholesterol is also reported to be enriched in the extracellular lipid leaflet and in lipid ordered domains of plasma membranes [43]. In the vesicles probably a more scrambled orientation of all reconstituted sperm lipids has taken place, which could form an explanation for the different composition of oxysterols in oxidation-stressed vesicles compared with sperm.

Effect of the Freeze/Thaw Procedure on Oxysterol Formation

In animal reproduction, freeze/thaw of sperm is common practice to store and distribute sperm before its use in artificial insemination. Freeze/thaw of sperm is also frequently performed in human medicine, for example, when a donor has to undergo chemotherapy, which typically has adverse effects on sperm quality. Analogous to screening the effect of storage in food products for oxysterols [13–17] and the knowledge that the freeze/thaw procedure is known to induce lipid peroxidation in semen [9, 44, 45], we investigated whether the formation of oxysterols was induced in sperm cells that had undergone this treatment. However, frozen/thawed sperm cells did not show an increase in oxysterol levels upon incubation. This is different from the observations made with the fluorescent peroxidation reporter C11-Bodipy$^{581/591}$ in sperm, where an increased peroxidation was observed under these conditions [9]. Several reasons may cause this difference, such as a different reactivity of cholesterol and the Bodipy probe toward various ROS. Both molecules are oxidized by hydroxyl radicals and peroxyl radicals, as well as by peroxynitrite [6, 46, 47]. However, whereas the reactivity of C11-Bodipy$^{581/591}$ toward ROS is comparable to that of polyunsaturated fatty acids, such as arachidonic acid [48], the reactivity of (monoenoic) cholesterol is considerably lower [6]. Furthermore, it is known that lipid peroxidation is not homogeneously distributed over the sperm cell, but that the mid piece of sperm (the only area of the sperm cell where mitochondria reside) is particularly prone to oxidation [9]. Cholesterol, however, is enriched in the sperm head cell plasma membrane, where relatively little lipid peroxidation was observed, which offers another explanation for the lower level of peroxidation observed with cholesterol [9, 20].

Oxysterol Formation in and Depletion from Sperm Cells

Interestingly, fresh sperm incubated in capacitation media did give rise to the formation of oxysterols under conditions that are routinely used for in vitro fertilization of mammalian oocytes (i.e., under conditions where sperm function and integrity lead to fertilization [18]). Under in vitro capacitation conditions, in which the medium is enriched in defatty bovine serum albumin, a complete depletion of formed oxysterols into the albumin-enriched medium was noted after 4 h of incubation. The capacitated sperm were virtually devoid of oxysterols, and thus of a fraction of the original cholesterol. Remarkably, the same treatments to frozen/thawed sperm did not result in the formation of oxysterols or in the albumin-mediated depletion of cholesterol (and oxidized products). Many studies have been performed that suggest that cytotoxicity of oxysterols may result from the induction of apoptosis [15, 49–57]. Sperm cells themselves appear to be well protected for apoptosis because of the absence of cytosol (containing the apoptotic machinery) and the hypercondensation of DNA (preventing fragmentation [58]), but sperm cells loaded with oxysterols could turn out to be poor matches for the egg cell they fertilize. Given the fact that oxysterols are potentially cytotoxic and the fact that sperm capacitation depends on proper sterol extraction, we hypothesize the following: The formation of oxysterols is a part of the ROS-induced initiation of sperm activation and subsequently allows albumin to deplete oxysterols from the sperm surface. It is possible that albumin has a greater affinity for the more hydrophilic oxysterols compared with cholesterol itself, which should be more difficult to extract from the membrane lipid bilayer, but this needs to be further investigated. At any rate, the depletion serves two goals: 1) scavenging of oxysterols from the sperm surface and 2) net depletion of sterols, thus allowing enhanced membrane fluidity, which is thought to be required for sperm capacitation. The fact that endogenous oxysterol formation was not induced by in vitro capacitation treatments of frozen/thawed sperm is interesting. It might show that either some of the ROS signaling pathways involved in sperm capacitation have been lost during the freeze/thaw process or that scavenging of ROS is better functional in fresh and frozen/thawed sperm compared with capacitated sperm. Alternatively, cholesterol ordering in the frozen/thawed sperm cells might be changed, which makes cholesterol less vulnerable for ROS. The lack of formation and removal of oxysterols may form a part of the explanation of why frozen/thawed sperm have lower fertilization potential compared with fresh sperm.

Future research should focus on the importance of oxysterol formation and subsequent albumin-mediated depletion during sperm capacitation. The relationship between oxysterol formation and sperm motility (signs of sperm capacitation in the tail) as well as sperm-zona interactions and acrosome responsiveness after such binding (signs of sperm capacitation on the sperm head) should be elucidated in such studies. Also, the role of vitamin E (which has been shown to inhibit bovine in vitro fertilization results [58, 59]) in eventually inhibiting oxysterol and its subsequent removal from the sperm surface formation needs to be addressed. Interestingly, in this light a recent study has shown vitamin E levels to correlate with an inhibition of both oxysterol formation and lipoprotein-mediated uptake of sterols in Caco-2 cells [60]. If a similar scenario is valid for sperm, it may provide us with a more fundamental understanding of how cholesterol is depleted from capacitating sperm, which for two decades has been known as a classical sperm capacitation response required for sperm to become competent to fertilize.

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