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# Trisialoganglioside GT1b Prevents Increase in Sperm Membrane Molecular Ordering Induced by In Vitro Lipid Peroxidation

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**ABSTRACT:** The effect of various types of gangliosides, the sialic acid–containing glycosphingolipids, on human sperm membrane during lipid peroxidation induced by $Fe^{2+}$/ascorbate ions was investigated. The monosialoganglioside (GM1), disialogangliosides (GD1a and GD1b), and trisialoganglioside (GT1b) were examined at a concentration of 100 μM, which was above their respective critical micellar concentrations. Lipid peroxidation was determined by quantification of malondialdehyde (MDA) concentration. The molecular orientational order in the membrane was assessed by fluorescence spectroscopy and electron paramagnetic resonance spectroscopy. Both approaches revealed a significant increase in membrane rigidity following oxidation, which correlated with an increase in the MDA level. The preincubation of spermatozoa with GM1 and GD1a did not have any effect on induced lipid peroxidation. In the presence of GD1b and GT1b, a reduced formation of MDA and a decrease in membrane rigidity was detected. The inhibitory effect of GT1b micelles toward membrane oxidation damage was found to be greater than that of GD1b. In conclusion, a direct relationship between the reduced content of the accumulated MDA and the longer preservation of the native-like membrane molecular ordering during sperm oxidation in the presence of GT1b suggests its protective effect. This phenomenon could be due to the specific GT1b conformation and its negative surface potential.

Key words: Membrane rigidity, gangliosides, spermatozoa.
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As in other cell types, lipid peroxidation in human spermatozoa is initiated by a variety of free radicals to which polyunsaturated fatty acids from cell membranes are exposed. The radicals involved in these processes are mainly oxygen centered and include superoxide, hydroxyl, and peroxyl radicals. Once initiated, the peroxidation cascade does not progress rapidly but is impeded, leading to accumulation of lipid peroxides in the sperm plasma membrane (Jones and Mann, 1976; see Saleh and Agarwal, 2002, for a review). A subsequent introduction of a ferrous ion promoter would induce the decomposition of lipid peroxides, stimulating a peroxidation chain reaction and resulting in the generation and release of further lipid peroxides from which malondialdehyde (MDA) is derived (Aitken et al, 1993a; Storey, 1997). Associated with these changes in the cell membrane, an increase in phospholipid bilayer rigidity on lipid peroxidation has been reported (Aitken, 1994; Ochsendorf et al, 2000). The physiologic importance of modifications in the physical properties of membranes resides in their relation to numerous cellular functions. Extensive membrane lipid peroxidation (LPO) results in a loss of membrane fluidity and a concomitant loss of sperm function (Aitken et al, 1993b; Alvarez and Storey, 1995). Only a few reports on the application of physical methods for investigating membrane fluidity in human spermatozoa (Giraud et al, 2000; Ochsendorf et al, 2000; Force et al, 2001), as well as on the influence of some agents in preventing changes in membrane fluidity induced by free-radical attack (Nivsarkar et al, 1996; Christova et al, 2004), have been published. Several agents are known to be stabilizers or protectors of cell membranes due to their inhibitory effects on lipid peroxidation (Bondy et al, 1990; Garcia et al, 2001; Sikka, 2004). Evidence has recently been provided that exogenously added gangliosides, the sialic acid–containing glycosphingolipids, are efficient in the protection from oxidative damage in a variety of cells. The ability of various types of gangliosides to diminish lipid peroxidation end-product accumulation and to exhibit the scavenging action against free radicals has been described in isolated heart cells (Maulik et al, 1993), brain cells (Bondy et al, 1990; Tyurin et al, 1992; Tyurina et al, 1993; Avrova et al,

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2002; Yamamoto and Mohanan, 2003), and low-density lipoproteins (Kveder et al, 2003). It has been found that gangliosides, added exogenously, improve the viability of preimplantation of mouse embryos after cryoconservation (Sakharova et al, 1997).

In our previous study, we reported on the protective effect of brain-ganglioside mixture in a model of induced lipid peroxidation in human spermatozoa (Kveder et al, 2004). In addition, we have shown, based on the biochemical approach, that different types of gangliosides exhibited different protective efficiencies in suppressing MDA formation during cell oxidation (Gavella et al, 2005). Determination of MDA content provides only an indirect measure of lipid peroxidation without the subcellular resolution of the membrane changes (Ball and Vo, 2002). Therefore, the aim of this study was to apply fluorescence and electron paramagnetic resonance spectroscopy (EPR) to gain an insight into the changes in the molecular ordering of a cell membrane exposed to the oxidation process. The extrinsic reporter groups (1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene p-toluene sulphonate [TMA-DPH] as a fluorescence probe and stearic fatty-acid, spin-label derivative for EPR) that partition in the surface lipid part of the membrane bilayer were incorporated into the spermatozoa. The induced oxidation was measured in the presence or absence of exogenously added different types of gangliosides. Correlation of the MDA formation with the change in the physical state of the sperm membrane was determined in the presence of ganglioside GT1b.

## Materials and Methods

### Media and Chemicals

Purified bovine brain individual gangliosides (monosialoganglioside GM1, disialogangliosides GD1a and GD1b, and trisialoganglioside GT1b) and ascorbic acid sodium salt were from Sigma Chemical Co (St Louis, Mo). The fluorescent probe TMA-DPH was purchased from Molecular Probes (Eugene, Ore). The spin label 5-doxylstearic acid nitroxide (5-NS) was synthesized according to Hubbell and McConnell (1971). Bioxytech LPO-586 assay kit was from Oxis Internationl (Portland, Ore). The ferrous sulphate and other chemicals were of analytic grade (Kemika, Zagreb, Croatia).

All the experiments were performed in $Ca^{2+}$ free and $Mg^{2+}$ free Hank balanced salt solution (HBSS; pH 7.4) because the presence of these divalent cations was found to suppress the level of lipid peroxidation (Aitken et al, 1993a).

### Oxidation of Cells

The specimens were collected from men undergoing routine fertility evaluation and characterized according to the *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction* (World Health Organization, 1999). The study was approved by the institution's ethics committee, and written informed consent was obtained from the participating subjects. The semen samples were diluted with HBSS and the cells were sedimented by centrifugation at $500 \times g$ for 5 minutes. The supernatant was discarded and the sperm pellet was resuspended gently in buffer and recentrifuged. To provide a sufficient number of sperm cells for all experiments, only the cell suspensions with a concentration higher than $50 \times 10^6$ cells/mL were taken into account.

Lipid peroxidation was induced in sperm suspension ($20 \times 10^6$ in 1 mL HBSS) according to a somewhat modified method of Aitken et al (1993a) using 66 μM of ferrous ion and 166 μM of ascorbate (final concentrations) and incubating the sample with gentle shaking at 37°C for 30 minutes.

In the experiments with gangliosides, the cells were preincubated with gangliosides (at 37°C for 15 minutes) prior to the introduction of the peroxidation promoter system. The control sample without the promoter system followed all the experimental stages in parallel. The gangliosides used in the experiments (ie, GM1, GD1a, GD1b, GT1b) were of the same final concentration of $1 \times 10^{-4}$ M, which was above their respective critical micellar concentrations of $(2 \pm 1) \times 10^{-8}$ M, $(2 \pm 1) \times 10^{-6}$ M, $(1 \pm 0.5) \times 10^{-6}$ M, and $(1 \pm 0.5) \times 10^{-5}$ M, respectively, at pH = 7.4 and 20°C (Ulrich-Bott and Wiegandt, 1984).

### Malondialdehyde Measurement

Malondialdehyde accumulation was spectrophotometrically measured using a specific Bioxytech LPO 586 test based on the reaction of the chromogenic reagent N-methyl-2-phenyl indole at 45°C to yield a stable chromophore monitored at 586 nm using an SP-8 spectrophotometer (Pye Unicam, Cambridge, United Kingdom) (Gomez et al, 1998). Malondialdehyde concentration was used as an index of lipid peroxidation of membranes and defined as μM of MDA formed by $10^8$ spermatozoa after incubation at 37°C for 30 minutes.

### Incorporation of Reporter Groups and Spectroscopic Measurements

Following oxidative treatment, the cells were washed carefully ($500 \times g$ for 5 minutes) in HBSS prior to the introduction of the reporter molecules to remove all extracellular species that might interfere with their incorporation into the sperm cell membrane.

*Fluorescence Spectroscopy*—The extrinsic fluorescent probe TMA-DPH bears a cationic trimethylammonium substituent in the DPH molecule, which acts as a surface anchor and improves the localization of DPH in the membrane in an orientation parallel to the long axis of the phospholipid molecules. The incorporation of TMA-DPH into spermatozoa was performed according to a slightly modified method by Giraud et al (2000). Briefly, the washed spermatozoa were suspended at a final concentration of $1 \times 10^6$ cells/mL in HBSS containing TMA-DPH (final concentration of 1 μM prepared from a stock solution of 0.3 mM in ethanol). The suspension was gently shaken and then incubated in the dark at room temperature for 5 minutes. Cell suspensions containing no TMA-DPH (blanks) were similarly assessed to correct for light scattering/turbidity (Lentz et al, 1979). Membrane fluidity of spermatozoa was assessed by evaluating

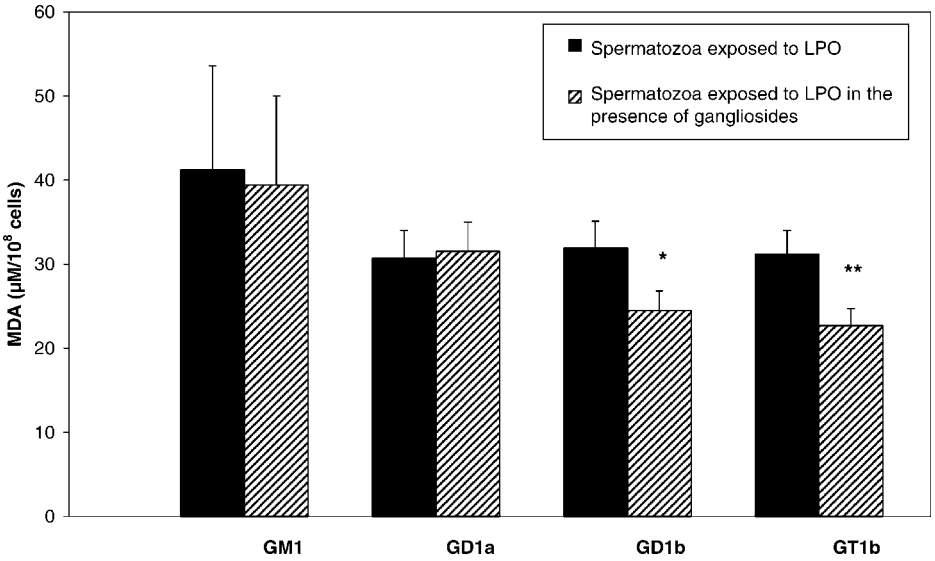


Figure 1. The effect of individual gangliosides (GM1, GD1a, GD1b, and GT1b) on malondialdehyde generated in human sperm cells exposed to induced lipid peroxidation at 37°C for 30 minutes. Data are means ± SEM (n = 5 for GM1 and GD1a, n = 13 for GD1b, and n = 16 for GT1b). Concentration of gangliosides was 100 μM. Statistical difference between the results from the samples containing gangliosides vs those without them (the control samples) was determined using Wilcoxon signed rank test. *$P < .002$; **$P < .0005$.

the steady-state fluorescence anisotropy of TMA-DPH incorporated into the cells, which can reflect the rotational diffusion of the fluorophore during the lifetime of the excited state (Lakowicz, 1983). The measurements were performed using a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave Victoria, Australia) with the 10-nm bandwidths of both the excitation (355 nm) and emission (428 nm) monochromators. Samples in 3-mL quartz cuvettes were excited with vertically polarized light, and vertically ($I_{vv}$) and horizontally ($I_{vh}$) polarized fluorescence intensities were measured using L-geometry optical path. The anisotropy (r) was calculated as $r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$. The correction factor (G), given as $G = I_{hv}/I_{hh}$, was determined by measuring the vertically ($I_{hv}$) and horizontally ($I_{hh}$) polarized fluorescence intensities using the horizontally polarized exciting light.

*EPR Spectroscopy*—The cells were spin labeled using 5-NS as previously described (Ochsendorf et al, 2000; Kveder et al, 2004). In brief, the pellet was resuspended in 200 μL of buffer in a glass tube that contained 5-NS deposited as a thin dry film on the glass wall (10 nmol of the spin label per $5 \times 10^7$ cells). Following the incorporation of the spin label (at room temperature for 30 minutes), the samples were pelleted using Beckman table-top centrifuge ($600 \times g$ for 1 minute), the supernatant was removed, and the pellet was resuspended in 1 mL of fresh buffer. The washing cycle ($600 \times g$ for 1 minute) was repeated twice to ensure the complete removal of the excess spin label. The pellet was then resuspended in 25 μL of the buffer and soaked in the EPR capillary (1-mm inner diameter). The EPR measurements were performed on an X-band Varian E-109 EPR spectrometer (Varian, Palo Alto, Calif). Data were collected using the supplied software (Morse, 1987). The spectra were recorded at 25°C with 10-mW microwave power, 0.1-mT modulation amplitude, and 100-kHz modulation frequency. The EPR experimental spectra were evaluated in terms of the apparent maximal hyperfine splitting, $2A_{max}$.

### *Statistical Analysis*

Statistical analysis was performed using a statistical package (Complete StatSoft CSS, Tulsa, Okla). The data are expressed as arithmetic mean and SEM values. In view of the non-Gaussian distribution of data, the nonparametric Wilcoxon signed rank test was employed to determine differences between the control and LPO-induced samples from the same spermatozoa.

## *Results*

The biochemical characterization of the induced spermatozoa oxidation is presented in Figure 1. Spontaneous MDA production in control samples was at a very low rate (ie, $<1 \mu M/10^8$ cells). However, MDA concentration was significantly raised in the presence of ferrous ions promoter (up to the factor of 40), indicating that membrane lipid peroxidation was induced. To compare the efficiency of different types of gangliosides in decreasing MDA accumulation on induced LPO, cells were preincubated with exogenously loaded gangliosides containing 1 (GM1), 2 (GD1a and GD1b), or 3 (GT1b) sialic acid residues. In all experiments, the concentration of gangliosides exceeded their respective critical micellar concentrations. Gangliosides GM1 and GD1a did not show any influence on MDA production during cell oxidation, whereas the protective effect of GT1b against MDA formation was found to be greater ($31.2 \pm 2.7$ vs $22.7 \pm 1.9$ $\mu M/10^8$ cells in the absence and presence of GT1b, respectively; n = 16; $P < .0005$) than that of GD1b ($31.9 \pm 3.1$ vs $24.5 \pm 2.3$ $\mu M/10^8$ cells in the absence and presence of GD1b, respectively; n = 13;

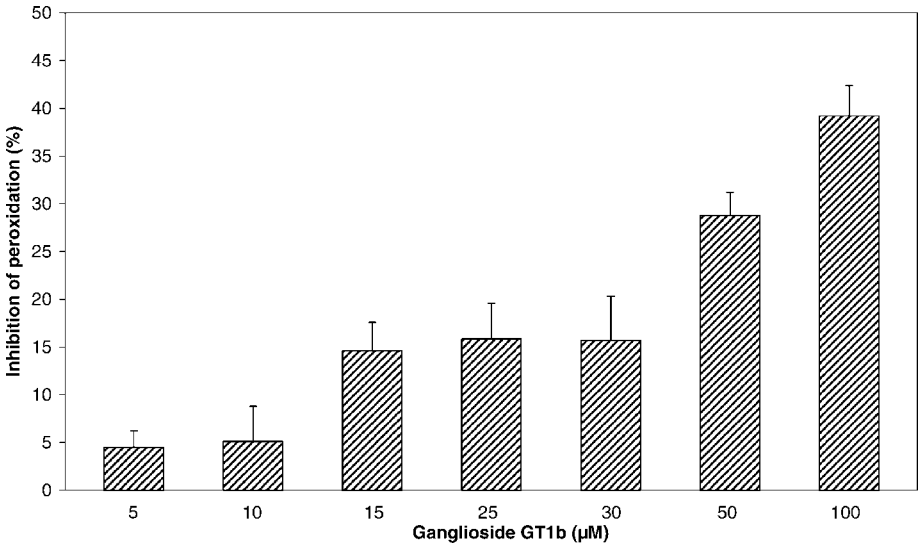


Figure 2. Dose-dependent effect of ganglioside GT1b on the inhibition of lipid peroxidation in human spermatozoa exposed to $Fe^{2+}$/ascorbate promoter system at 37°C for 30 minutes. Data have been expressed as the percentage of inhibition of lipid peroxidation in the presence of GT1b. Data are means ± SEM (n = 4 in the experiments with 5-, 10-, 15-, and 25-μM GT1b; n = 8 for 30-, 50-, and 100-μM GT1b).

$P < .002$). Hence, only GT1b was used in further experiments. Figure 2 presents the dose-dependent effects of GT1b ranging from 5–100 μM on MDA generated in sperm cells exposed to oxidation promoter. To eliminate the variability in the absolute values of MDA in different sperm samples, data have been expressed as the percentage of inhibition of lipid peroxidation in the presence of GT1b. The results indicated that the preincubation of spermatozoa with GT1b at a concentration higher than 50 μM significantly reduced MDA formation compared with the cells oxidized in the absence of GT1b. As the addition of 100-μM GT1b decreased the MDA formation by approximately 40%, this concentration was used in further experiments.

To study changes in the physical state of the sperm membrane exposed to oxidation, the molecular ordering was assessed using fluorescence spectroscopy (Figure 3A). The comparison of the steady-state fluorescence anisotropy revealed a significant increase in the anisotropy for oxidized vs nonoxidized cells, implying a decrease in molecular orientational freedom in the oxidized sperm membrane. In nonoxidized cells, a decrease in the anisotropy was observed in the presence of GT1b. At the same time, fluorescence emission in these samples was increased in a concentration-dependent manner (Figure 3B). This finding indicated a different environment probed by the reporter group in the samples loaded with gangliosides with respect to those not exposed to them. The steady-state fluorescence anisotropy in samples loaded with gangliosides exposed to oxidation was found to increase in comparison with the nonoxidized samples. The EPR spectroscopy was applied to independently probe the molecular ordering in sperm membrane exposed to oxidation (Figure 4). An increase in the apparent hyperfine splitting in the spectra of oxidized cells related to a motionally more restricted environment experienced by the reporter group was observed, whereas oxidation-induced spectral changes were found to be suppressed in the presence of GT1b. These results can be attributed to the MDA formation as presented in the Table. A statistically significant difference in the apparent maximal hyperfine splitting was observed between the samples oxidized in the presence of gangliosides vs those oxidized in their absence, $2A_{max}$ and MDA content being smaller in the ganglioside-treated samples. Interestingly, no significant difference in $2A_{max}$ was observed between the control samples and those incubated with gangliosides only.

## Discussion

In our previous study, we reported on the ability of gangliosides to reduce the accumulation of lipid peroxidation end products in the sperm membrane (Gavella et al, 2005). In this investigation, we examined the micellar inhibitory effect of gangliosides (GM1, GD1a, GD1b, and GT1b) on sperm membrane molecular ordering induced by iron/ascorbate lipid peroxidation. The approach was based on the combination of biochemical determination of MDA production and spectroscopic fluorescence and EPR methods.

The results obtained by fluorescence anisotropy measurements using TMA-DPH showed that sperm membrane rigidity increased during induced lipid peroxidation.

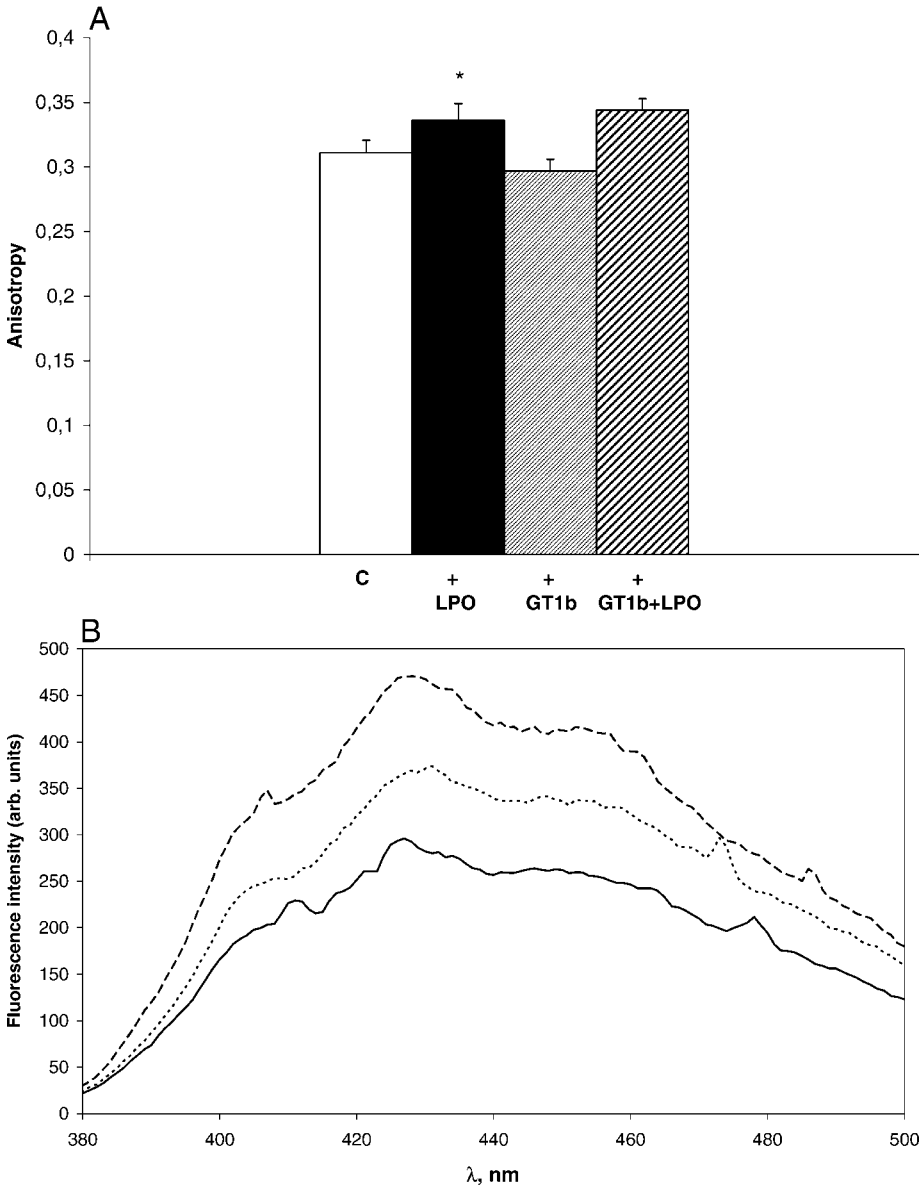


Figure 3. **(A)** The steady-state fluorescence anisotropy of (1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene p-toluene sulphonate (TMA-DPH) in different sperm cell samples. □ indicates control sample (ie, nonoxidized without GT1b; (C); ■ sample exposed to lipid peroxidation (+LPO); ⬚ sample loaded with GT1b (+GT1b); ▨ sample loaded with GT1b and exposed to LPO (+GT1b +LPO). Data are means ± SEM (n = 5). Concentration of GT1b was 100 μM. *$P < .05$ in comparison with control samples according to Wilcoxon signed rank test. **(B)** The fluorescence emission spectra (excitation at 355 nm) of TMA-DPH are presented for the control sample (solid line) and sample exposed to 10 μM (dotted line) or 100 μM GT1b (dashed line).

This finding is consistent with the reports that studied different biologic systems exposed to oxidation applying different fluorescent probes/methods (Garcia et al, 2001; Ball and Vo, 2002; Bhosle et al, 2002). The observation was independently supported by the results obtained by EPR spectroscopy, where the increase in the apparent hyperfine splitting in oxidized samples could be directly related to the decrease in motional degrees of freedom of the 5-NS reporter group bearing the paramagnetic center at the level of fifth C-atom with respect to the lipid-water interface (Ochsendorf et al, 2000; Kveder et al, 2004). This could be explained by the oxidation of fatty acid double bonds that are one of the key factors in the control of functional membrane fluidity. Once the fatty acids are damaged and the products of lipid peroxidation formed, the changed membrane matrix imposes mobility restrictions, as experienced by the reporter group. The chemical modifications of the constituent molecules result in the perturbation of molecular interactions/packing determining the physical state of the membrane. This reasoning

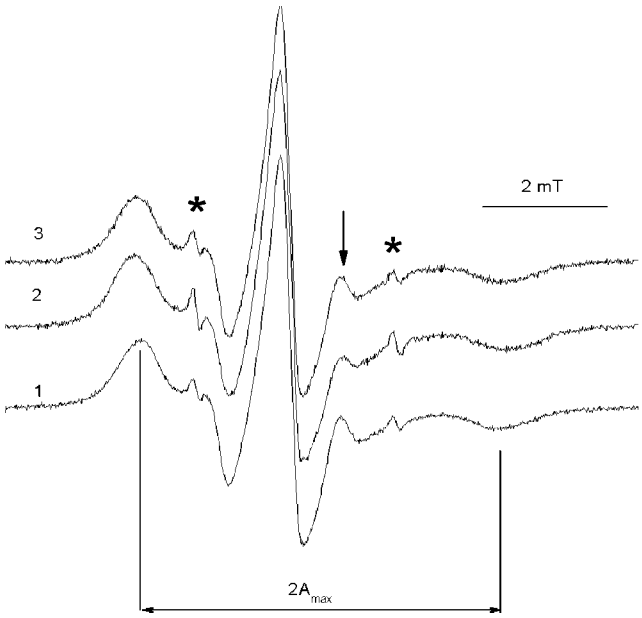


Figure 4. The electron paramagnetic resonance spectra of sperm plasma membrane spin labeled with 5-doxylstearic acid nitroxide. The spectra are normalized to the same maximum amplitude and denoted as follows: 1 indicates control sample (ie, not oxidized); 2, sample oxidized in the absence of gangliosides; and 3, sample oxidized in the presence of GT1b. The maximal hyperfine splitting ($2A_{max}$) is indicated on the spectrum of the control sample. The spectral component denoted with "*" is attributed to the spin label not incorporated into the cell membrane. The peak arrowed in the figure is sensitive to the motional status of the nitroxide reporter group. Spectra presented here belong to the specimen from the same donor.

*The effect of sperm cell incubation with ganglioside GT1b prior to the induction of LPO on membrane molecular ordering monitored by electron paramagnetic resonance spectroscopy ($2A_{max}$) and MDA concentration**

| Sperm Samples | $2A_{max}$ (mT) | MDA ($\mu M/10^8$ cells) |
|---|---|---|
| Control | 5.58 ± 0.05 | 0.8 ± 0.6 |
| Control with GT1b | 5.53 ± 0.05 | 0.7 ± 0.5 |
| Oxidized sample | 5.77 ± 0.07[a] | 22.9 ± 3.1[b] |
| Sample oxidized in the presence of GT1b | 5.65 ± 0.07[c] | 14.5 ± 2.4[c] |

* Data are means ± SEM (n = 7). Concentration of GT1b was 100 μM. Statistical significance was determined using Wilcoxon signed rank test. [a] and [b] indicate statistical difference vs control ($P < .01$ and $P < .0001$, respectively); [c], statistical difference vs oxidized samples ($P < .01$). LPO indicates lipid peroxidation; and MDA, malondialdehyde.

was supported by the direct correlation of MDA production, with the increase in the fluorescence anisotropy or hyperfine splitting observed in the EPR spectra.

Based on the results of the experiments aiming to show the protective role of an individual monoganglioside, disialogangliosides, or trisialoganglioside by fluorescence spectroscopy, an unambiguous conclusion could not be drawn. Namely, the TMA-DPH probes different environments in the presence vs absence of gangliosides already in the nonoxidized samples. The increase in the fluorescence emission, together with a decrease in the anisotropy in the presence of gangliosides in the nonoxidized samples, might be explained by a direct interaction of TMA-DPH with gangliosides or ganglioside micelles anchored at the surface of the cell competing with fluorophore partitioning into the membrane bilayer. This was supported by an intense fluorescence of the pure solution of gangliosides with TMA-DPH (data not shown), described in the literature as an indication of a direct interaction of a fluorophore with gangliosides (Ravichandra and Joshi, 1999). Because of these difficulties in the interpretation of the fluorescence data in the presence of gangliosides, the EPR spectroscopy was applied. The spectra of 5-NS were used to study the ability of gangliosides to reduce the $Fe^{2+}$-mediated decomposition of lipid hydroperoxydes from the sperm membrane. The spectral analysis indicated that the oxidation-induced changes were significantly suppressed in the presence of trisialoganglioside GT1b, as confirmed by a significantly reduced MDA formation in these samples. A less-pronounced protective effect was obtained with disialoganglioside GD1b, whereas no effect was observed with disialoganglioside GD1a or monosialoganglioside GM1.

To explain the observed phenomena, the nature of the association between exogenously added gangliosides and sperm plasma membrane should be discussed. Knowledge gained on other cell types and model systems, including synthetic ganglioside analogs (bearing, for example, spin-label tags), could be extrapolated to this study (Sharom and Ross, 1986; Ravichandra and Joshi, 1999). The action of the gangliosides on cell membrane could be explained by their self-aggregation in aqueous media into micelles of different shapes and sizes that are strongly influenced by the sialic acid residues (Ulrich-Bott and Wiegandt, 1984; Schwarzmann, 2001; Yokoyama et al, 2001). On addition to cells, exogenous gangliosides might be loosely bound to the cell surface, some fraction of ganglioside micelles might be tightly attached, and some ganglioside monomers would be expected to be inserted into the outer leaflet (Schwarzmann, 2001). In this study, individual gangliosides showed different abilities to protect sperm membrane during lipid peroxidation. The most pronounced effect was obtained with trisialoganglioside GT1b micelles, which bear the highest absolute negative surface potential. Due to this property, GT1b could act as a chelator of ferrous ions, as shown in our previous biochemical study that revealed a similarity in the mechanism of LPO inhibition by a comparison with EDTA (Gavella et al, 2005). Because in the presence of either EDTA or GT1b the MDA content was diminished and the oxidative modifications of sperm cells was suppressed, the membrane ordering was found to be preserved based on spectroscopic data (data not shown). It

should be stressed that EDTA is a membrane-impermeable chelator, for which reason its chelating action is restricted to the extracellular space. However, various modes of ganglioside binding/incorporation to the cell membrane, particularly at concentrations exceeding their respective critical micellar concentration, should also be taken into account. It cannot be excluded that gangliosides anchored in the membrane interact with their polar head groups with the epitopes exposed at the surface of the cell. The propagation of the oxidation induced from the extracellular space toward the interior of the membrane, where polyunsaturated fatty acids oxidation takes place, is thus prohibited. As a support of this reasoning a specificity of the ganglioside type, not only the number of sialic acid–bearing residues governing the interaction with the cell surface, can be offered.

In this framework, the less-pronounced protective effect of GD1b, and no effect of GD1a or GM1, might indicate that the position and linkage type of sialic acid on the nonreducing end of galactose are also important. The latter might influence the exposure of negative charges in ganglioside micelles differing in the architecture or in the membrane-incorporated ganglioside monomers, thus having an impact on the interaction with positively charged ferrous promoter systems. This is supported by the fact that the second sialic acid on the nonreducing part of galactose was in the $\alpha 2 \rightarrow 8$ position in both GT1b and GD1b (Vasudevan and Balaji, 2002). Contrary to this, GD1a and GM1, having sialic acid in the $\alpha 2 \rightarrow 3$ position, did not exhibit any effect on sperm cells in either biochemical or EPR measurements.

In conclusion, we have demonstrated that GT1b had the greatest ability among the studied gangliosides to preserve the native molecular ordering in the sperm membrane exposed to oxidative stress when used above its critical micellar concentration. GD1b showed a lesser protective ability than GT1b, whereas GD1a and GM1 exhibited no protective effect whatsoever. The obtained results suggest that GT1b prevents lipid peroxidation–induced sperm membrane damage due to its specific molecular structure, thus acting as a ferrous ion chelator and/or a sterical shield of the reactive epitopes at the cell surface.

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