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Effect of High Pressure and Sub-Zero Temperature on Total Antioxidant Capacity and the Content of Vitamin C, Fatty Acids and Secondary Products of Lipid Oxidation in Human Milk

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The objective of this study was to compare of the effects of high pressure of 193 MPa at -20°C and Low Temperature Long Time pasteurization (LTLT or holder pasteurization, 62.5°C, 30 min) on the content and composition of fatty acids (FAs), concentrations of secondary products of lipid oxidation (TBARS), the total antioxidant capacity (TAC), total vitamin C and ascorbic acid (AsA) content in human milk. It was shown that no significant changes in the content and composition of FAs and TBARS levels were noted in both pressurized and LTLT pasteurized milk samples. The results obtained indicate that the antioxidant properties in pressurized human milk were also not affected. In the case of the pasteurized samples only slight (approx. 6%) and statistically insignificant decrease was observed in the Trolox equivalent antioxidant capacity (TEAC) values. Pasteurization significantly reduced the content of total vitamin C and AsA, by 35% and 24%, respectively. A minor and statistically insignificant (approx. 6%) decrease in vitamin C levels was observed in milk treated with high pressure. However, a significant decrease (by more than 11%) occurred in these conditions in AsA concentrations. The influence of high pressure treatment on AsA levels and the lack of significant changes in TEAC values point to the relative stability of the remaining antioxidant components in human milk. Further research is needed to determine the effects of high pressure of approximately 200 MPa and sub-zero temperatures on, mainly thermolabile, components of human milk, which are degraded by LTLT pasteurization.

INTRODUCTION

Breastfeeding is the best way to feed a newborn and a small child. Human milk is characterized by an optimal quantitative and qualitative balance of nutrients which are essential for healthy growth at every stage of development. Newborns, in particular premature infants, should be fed human milk from the corresponding lactation phase. If, for some reason, a child cannot be fed orally or with the mother's milk, nourishment that is most appropriate in a given stage of development can be obtained from a human milk bank (HMB). In HMB, milk is treated by Low Temperature Long Time (LTLT) pasteurization at 62.5°C for 30 min, which is also known as holder pasteurization. Pasteurization minimizes the risk of transfer of pathogenic agents such as cytomegalovirus, hepatitis B, HIV and microbial infections which are particularly dangerous for premature infants. However, LTLT contributes to significant degradation of thermolabile bioactive components and decreases the nutritional value of human milk [Van Gysel *et al*., 2012; Tully *et al*., 2001].

Lipids, including polyunsaturated fatty acids (PUFAs), are vital components of human milk. In addition to the precursors of n-6 and n-3 long-chain PUFAs (LC-PUFAs), namely linoleic acid (LA) C18:2n-6 and α-linolenic acid (ALA) C18:3n-3, human milk also contains the metabolites of both fatty acid (FA) families. The most nutritionally important FAs in human milk are: arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). The demand for LC-PUFAs is determined by their role in the development of the nervous system and the retina [Stolarczyk & Socha, 2002; Januszewicz *et al*., 1999]. In infants, the enzymatic system is not yet fully developed, and is incapable of producing sufficient quantities of LC-PUFAs which have to be supplied in the diet [Kent *et al*., 2006; Innis *et al*., 1999; Emmett *et al*., 1997].

Human milk lipids are characterized by high PUFA levels, which makes them susceptible to oxidation. The degree of lipid oxidation can be determined based on the concentrations of secondary oxidation products [Esterbauer *et al*., 1991; Turoli *et al*., 2004]. The human body needs antioxidant substances to maintain a state of homeostasis. Newborns, in particular premature infants, are exposed to powerful oxidative stress. Antioxidants present in the mother's milk eliminate excessive quantities of reactive oxygen species (ROS) from cells in a baby's body [Friel *et al*., 2004; Canfield *et al*., 2003].

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One of such antioxidants is vitamin C which is present in human milk in higher concentrations than other vitamins. Vitamin C is highly sensitive to external factors, and is one of the key indicators of changes in food products that are induced by processing [Moltó-Puigmartí *et al*., 2011; Siegel, 1993].

In view of the above, the preservation methods deployed by HMB should induce minimal changes in the nutritional composition of human milk. Continued efforts are made to develop new processing techniques that guarantee microbiological safety while preserving the nutritional and biological value of milk. One of the proposed solutions involves the use of high pressure to limit nutrient loss and preserve the product's sensory properties [Tewari *et al*., 1999] and selected biologically active components, such as lysozyme and peroxidase [Viazis *et al*., 2007; Mazri *et al*., 2012]. The bactericidal effects of high pressure are enhanced at below-zero temperatures [Hayakawa *et al*., 1998].

The objective of this study was to determine the effect of high pressure (193 MPa) applied at a temperature of -20°C on the content and composition of FAs, the degree of FA oxidation based on the concentrations of secondary products of lipid oxidation, the antioxidant capacity and vitamin C content of human milk, and to compare the above results with the outcomes of LTLT pasteurization.

All research procedures were approved by the Local Ethics Committee of the Medical University of Gdansk. The subjects gave their informed consent before the start of any procedure.

MATERIALS AND METHODS

Material

The experimental material comprised mature milk from seven healthy mothers, residents of Gdańsk, who gave birth to term babies at the Maternity Ward of the Clinical Hospital in Gdańsk. No complications were reported in any of the subjects in the perinatal period. All infants were born in good condition and with normal birth weight (3100–3800 g).

Milk collection

Milk was collected by the mothers with the use of an electric breast pump (Symphony, Medela) in accordance with general hygiene standards. It was collected within 24 h and refrigerated at 4°C. Milk samples were pooled and divided into 60 samples which were frozen at -80°C until analysis, but not longer than three weeks.

LTLT pasteurization

Glass vessels containing 20 mL of human milk each were placed in a water bath with a magnetic stirrer and heated at 62.5°C for 30 min. Pasteurized samples were quickly cooled to approximately 20°C and immediately analyzed.

High pressure treatment

Milk samples were subjected to high pressure generated in accordance with the procedure described by Malinowska-Pańczyk *et al*. [2009]. In the applied method, pressure was generating as a result of the increasing volume during ice formation in a sealed vessel filled with water kept at sub-zero temperatures. At a temperature of -20°C, the generated pressure equaled 193 MPa. Above this temperature, the sample placed in a sealed vessel is affected by the pressure in unfrozen state.

Isolation of fat from human milk

Fat was isolated from human milk by the modified extraction method described by Röse-Gottlieb in accordance with the Polish Standard [PN-EN ISO 1211:2011]. The procedure of removing solvent residues by drying the isolated fat at 102°C, recommended by the standard, was replaced with blowing the extracted fat to constant weight under a stream of nitrogen. The above procedure was applied to prevent the oxidation and degradation of FAs under the influence of high temperature, which is particularly important in PUFA analyses.

Preparation of fatty acid methyl esters

Fatty acids from samples of human milk fat were esterified in accordance with the Polish Standard [PN-EN ISO 12966–2:2011]. The esterification reaction was catalyzed by boron trifluoride (BF_3). The resulting solutions of fatty acid methyl esters (FAMEs) in hexane were stored at -20°C until analysis.

Analysis of FA composition by high resolution gas chromatography (HR-GC)

Fatty acid methyl esters were identified based on differences in the length of the carbon chain and the presence, number and configuration of double bonds, using a Hewlett-Packard gas chromatograph with a split-splitless injector and a flame ionization detector (FID) on an Rtx 2330 column (100 m × 0.25 mm) (Restek, Bellefonte, PA, USA). The carrier gas was helium with a flow rate of 0.6 mL/min. Injector and detector temperature was 250°C. The initial column temperature of 155°C was maintained for 30 min, after which it was increased at 1.5°C per minute to 210°C and held constant until the end of the chromatographic analysis. The analysis was completed within 120 min.

Quantitative and qualitative analyses of fatty acids were performed with the use of FAME reference solutions (Supelco Bellefonte, Pennsylvania, USA; Larodan Fine Chemicals, Malmö, Sweden) and the external standard method using correction factors (according to Polish Standard [PN-EN ISO 5508:1996]). The results for all identified FAs with a chain length of 6 to 24 carbon atoms and up to 6 double bonds were expressed as a weight percentage of total fatty acids.

Determination of the content of secondary products of lipid oxidation

The oxidation of PUFAs was evaluated by measuring the content of thiobarbituric acid reactive substances (TBARS) in human milk [Turoli *et al*., 2004]. To this end, 1 mL of 15% trichloroacetic acid (TCA), 0.6 mL of 0.25 mol/L HCl and 0.1 mL of 0.4% BHT solution in ethanol were added to centrifuge tubes containing 1 mL of milk samples. Tube contents were stirred and centrifuged at 6000×*g* for 10 min. Then, 0.6 mL of 0.375% TBA was added to the supernatant. The resulting mixture was saturated with

nitrogen, the tube was capped and heated in a water bath at 95°C for 30 min. The samples were rapidly cooled to stop the reaction, distilled water was added to the 4 mL mark, and the tubes were centrifuged at 6000×*g* for 5 min. Supernatant absorbance was measured at λ=534 nm against the reference. TBARS content was expressed as malondialdehyde (MDA) equivalent content based on a calibration curve showing the correlation between absorbance and MDA concentrations in standard solutions.

Determination of total antioxidant capacity

The total antioxidant capacity (TAC) of milk was determined with the use of the ABTS reagent [Martysiak-Żurowska & Wenta 2012]. Centrifuge tubes were filled with 2 mL of the $ABTS^{\bullet+}$ solution (A = 0.700 ± 0.002; measured relative to methanol at λ=734 nm), and 20 μL of milk were added. The mixture was stirred and incubated for 15 min at room temperature, without light access. After incubation, the mixture was centrifuged at 6000×*g* for 5 min, and the absorbance of the colored, clear supernatant was measured immediately at λ=734 nm relative to methanol. The total antioxidant capacity was measured as Trolox equivalent antioxidant capacity (TEAC) with the use of a calibration curve showing the correlation between absorbance and Trolox concentrations in standard solutions.

Determination of vitamin C content

Vitamin C content of milk was determined by the method described by Romeu-Nadal *et al.* [2006] which relies on reverse-phase high performance liquid chromatography with UV detection (RP-HPLC/UV). Vitamin C occurs in two forms: ascorbic acid (AsA) and dehydroascorbic acid (DHsA). To determine the total content of vitamin C, DHsA was reduced to AsA with the use of DL-dithiothreitiol (DTT). Degradation of AsA was prevented with the use of a 0.56% solution of metaphosphoric acid (V) ($H_3O_3P_9$) as a stabilizing agent. Then, 15% TCA was used to obtain clear solutions. Test tubes were filled with 300 μL of milk and 800 μL of 0.1 mol/L DTT. The mixture was shaken for 30 s and incubated at room temperature for 15 min, without light access. Afterwards, 300 μL of 0.56% $H_3O_3P_9$ and 20 μL of TCA were added, and the tubes were repeatedly shaken for 30 s. The mixture was transferred to filtration columns and centrifuged at 4500×*g* and 10°C for 10 min. The content of AsA was determined without DHsA reduction with DTT.

Vitamin C content of clear solutions was determined by RP-HPLC/UV in the Perkin Elmer liquid chromatography system with a C-18 column (Merck, Darmstadt, Germany) (125 × 4.0 mm I.D., 5 μm particle size) and a UV-VIS detector (λ=245 nm). The analyzed solution (25 μL) was applied to the column at a temperature of 25°C. The mobile phase was a mixture of 0.1% acetic acid and methanol (95:5, v:v). Vitamin C content (mg/100 mL), as the combined content of AsA and DHsA, and the AsA content of the analyzed milk samples were calculated with the use of a linear calibration equation expressing the relationship between the surface area of the generated peaks and the AsA content of standard solutions. Vitamin C recovery of the analytical method was taken into consideration in the final calculations.

Statistical analysis

The results were expressed as mean values with standard deviation. Data were processed statistically using Statistica 12.0 software. The significance of differences between the content of each analyte in raw human milk (RHM), pasteurized human milk (PHM) and high-pressured human milk (HPHM) was determined by one-way ANOVA and Tukey's post-hoc test. The results were regarded as significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Content and composition of fatty acids

The presence of more than 50 FAs, including *cis*- and *trans*-monoenic isomers of 14:1, 15:1 16:1 and 17:1 FAs, was determined in the analyzed human milk samples. The predominant FAs in human milk fat are: C18:1 (n-9) (36.5% of total FAs), C16:0 (25%), C18:2 n-6 (8.7%), C18:0 (5.7%), C14:0 (6.6%) and C12:0 (4.5%) (Table 1). The above FAs accounted for nearly 90% of the total fatty acids in human milk lipids. The remaining 10% was composed of LC-PUFAs such as AA, EPA, DPA and DHA, which are very important for infants, and bioactive CLA. Lipids extracted from the pooled milk sample (from 7 mothers) contained approximately 44% of saturated fatty acids (SFAs) and 43.5% of monounsaturated fatty acids (MUFAs). The content of PUFAs in the lipids of the pooled milk sample was estimated at 12% of the total fatty acids (Table 1).

No significant changes in fatty acid content were noted in milk samples subjected to the pressure of 193 MPa and temperature of -20°C. The observed changes in the proportions of FAs (SFA:MUFA:PUFA) in HPHM samples were not statistically significant ($p > 0.05$). The n-6/n-3 PUFA ratio in RHM samples was determined at 7.2:1, and it was not modified by high pressure treatment. In other studies, exposure to pressures as high as 400 MPa at above-zero temperatures (12–26°C) did not influence the content or composition of FAs in human milk fat either [Moltó-Puigmartí *et al*., 2011; Delgado *et al*., 2014]. The content of selected FAs in human milk, including C15:0, C18:3 n-3, C18:3 n-6, C20:0, C20:4 n-6 and C22:6 n−3, was significantly modified under exposure to 600 MPa, which led to considerable changes in the proportions of FAs, in particular a substantial drop in the levels of n-3 PUFAs [Delgado *et al*., 2014]. According to the authors, the significant decrease in PUFA levels under exposure to 600 MPa makes the resulting milk unsuitable for infants and suggests that human milk should not be treated at such high pressures.

The choice of lower pressures for breast milk preservation was also supported by the fact that high-pressure treatment at 600 MPa degraded IgA content by approximately 21% and the degree of tocopherol degradation was similar to that reported during holder pasteurization. The application of 400 MPa did not induce changes in the content of IgA or α- and δ-tocopherol [Delgado *et al*., 2014; Permanyer *et al*., 2010]. Despite the above, treatment at pressures lower than 600 MPa, especially at a temperature of 20–35°C could be ineffective in eliminating all microbial pathogens from human milk. The treatment's lethal effects on microbial activity can

TABLE 1. Composition of fatty acids in control samples (RHM) and samples pressurized at 193 MPa at the temperature of -20°C (HPHM) (% of total FAs ± SD).

Fatty acids	RHM (n=8)	HPHM (n=8)
C8:0	0.02±0.032	0.02±0.022
C10:0	0.80±0.089	0.78±0.081
C12:0	4.51±0.415	4.45±0.057
C13:0	0.01±0.018	0.01±0.016
C14:0	6.63±0.449	6.70±0.596
C14:1 9c	0.09±0.054	0.09±0.069
Other C14:1 c isomers	0.07±0.044	0.07±0.037
C15:0	0.36±0.051	0.35±0.050
C15:1 9c	0.08±0.013	0.07±0.015
Other C15:1 (c+ t) isomers	0.06±0.017	0.06±0.017
C16:0	24.97±1.309	25.61±1.233
Total C16:1 t	0.65±0.108	0.58±0.052
C16:1 9c	2.38±0.386	2.37±0.384
Other C16:1 c isomers	0.17±0.051	0.17±0.053
C17:0	0.26±0.012	0.26±0.013
C17:1 9c	0.04±0.011	0.04±0.012
Other C17:1 isomers	0.22±0.065	0.13±0.086
C18:0	5.67±0.385	5.79±0.481
Total C18:1 t	0.54±0.024	0.65±0.036
C18:1 (n-9)	36.55±0.845	36.2±0.558
C18:1 (n-7)	1.82±0.299	1.84±0.562
Other C18:1 c isomers	0.21±0.082	0.17±0.085
C18:2 tt	0.03±0.020	0.03±0.031
C18:2 (n-6) LA	8.87±0.784	8.59±0.855
C18:3 (n-6) GLA	0.03±0.020	0.03±0.012
C18:3 (n-3) ALA	0.96±0.057	0.91±0.067
C20:0	0.14±0.042	0.13±0.021
C20:1 (n-9)	0.59±0.041	0.65±0.062
Total CLA*	0.27±0.051	0.26±0.014
C20:2 (n-9)	0.35±0.116	0.36±0.117
C22:0	0.04±0.010	0.03±0.021
C20:3 (n-6) DGLA	0.32±0.084	0.31±0.137
C20:3 (n-3)	0.04±0.012	0.03±0.013
C22:1 (n-9)	0.24±0.069	0.23±0.068
C20:4 (n-6) AA	0.52±0.131	0.51±0.137
C20:5 (n-3) EPA	0.08±0.040	0.12±0.069
C22:2 (n-9)	0.02±0.010	0.02±0.019
C24:0	0.05±0.015	0.05±0.022
C24:1 (n-9)	0.09±0.025	0.08±0.031
C22:4 (n-6)	0.06±0.025	0.04±0.019
C22:5 (n-3) DPA	0.10±0.033	0.10±0.034
C22:5 (n-6)	0.18±0.088	0.18±0.094
C22:6 (n-3) DHA	0.22±0.091	0.21±0.091
SFA	43.46±2.293	44.18±2.006
MUFA	43.80±0.940	43.41±0.915
PUFA	12.03±1.690	11.69±1.695
PUFA n-3	1.39±0.462	1.36±0.538
PUFA n-6	9.97±1.478	9.66±1.457

CLA – conjugated linoleic acid.

be intensified by applying temperatures higher than 35°C or lower than 20°C [Pandya *et al*., 1995; Simpson & Gilmour, 1997; Reyns *et al*., 2000].

TBARS content

TBARS content expressed in terms of MDA is presented in Table 2. The content of TBARS in human milk is determined by various factors, including the mother's health and exposure to cigarette smoke [Ermis *et al*., 2005]. In the tested samples of raw human milk, TBARS levels were determined at 3.35 μmol/L MDA, and similar results were reported by Turoli *et al*. [2004]. A minor increase (8.7%) in TBARS concentrations was noted after LTLT pasteurization, but the observed change was not statistically significant. No changes in TBARS values were noted in the samples subjected to high pressure of 193 MPa at a temperature of -20°C. The insignificant rise in TBARS levels and the absence of changes in the content and composition of FAs under the influence of LTLT and high pressure (Table 1) indicate that the above processes do not stimulate the oxidation of LC-PUFAs. Human milk fat occurs in the form of fat globules surrounded by a layer of protein. The protein coat and the abundance of components with antioxidant properties in human milk effectively prevent fat degradation during high-temperature and high-pressure treatment.

Total antioxidant capacity

The Trolox equivalent antioxidant capacity (TEAC) of raw human milk, milk processed by LTLT pasteurization and milk subjected high pressure at sub-zero temperature is presented in Table 2. The TEAC value of RHM samples was estimated at 40 mg TE/100 mL. The results of the analysis indicate that high-pressure treatment at sub-zero temperature did not affect the antioxidant properties of human milk (Table 2). LTLT pasteurization induced a minor and statistically insignificant drop in TEAC values (by approx. 7%). Silvestre *et al*. [2008] measured the antioxidant capacity of human milk with the use of commercially available assay kits (PAO-DELTACLON, KPA-050 Cosmo Bio Co. Ltd.) and demonstrated a significant decrease in its values (by 66% on average) in milk heated at 63°C for 30 min. The assay measures the amount of the colored complex produced by the Chromatic Solution substrate (Bathocuproine) and Cu(I) ions reduced from Cu(II) by antioxidant substances in the sample. The differences in the results reported by Silvestre *et al*. [2008] and our findings could be attributed to different methods applied to determine the antioxidant properties of milk. The two analytical methods would have to be compared for the above assumption to be validated. According to Silvestre *et al*. [2008], their method supports the identification of both hydrophilic (vitamin C and glutathione) and hydrophobic antioxidants (vitamin E), but Prior *et al*. [2005] demonstrated that the method where Cu(II) ions are reduced to Cu(I) has certain limitations in determining the content of lipophilic substances with antioxidant properties (such as β-carotene). In our study, the ABTS reagent was used to determine the Trolox-equivalent antioxidant capacity of human milk which expresses the activity and content of all antioxidants in a given sample, including vitamins, enzymatic systems that scavenge free radicals, un-

TABLE 2. Thiobarbituric acid reactive substances (TBARS, μmol MDA/L), total antioxidant capacity (mg TE/100 mL), vitamin C and AsA (mg/L) concentrations in raw (RHM), LTLT pasteurized (PHM) and pressurized (HPHM) human milk.

	RHM (n=20)	PHM (n=20)	HPHM (n=20)
TBARS	3.35±0.64	3.64±0.70	3.25±0.68
TEAC	40.48±5.44	37.53 ±4.52	41.36±4.56
Vitamin C	34.37±4.56[a]	22.23±4.59[a,b]	32.29±4.82[b]
AsA	17.41±2.50[a]	13.27±2.53[a]	15.46±2.59[a]

The same letters in the same row indicate statistically significant differences (Tukey's test, $p < 0.05$).

known antioxidants, as well as the interactions between those components [Prior *et al*., 2005; Turoli *et al*., 2004].

Vitamin C and AsA content

Human milk is abundant in vitamin C, a powerful antioxidant. Vitamin C is sensitive to external factors such as temperature, light and pH, therefore, it is an effective indicator of changes induced by processing in food products, including human milk.

The content of total vitamin C, expressed as the combined content of AsA and DHsA, and the content AsA were determined in samples of raw human milk, milk treated by LTLT pasteurization and milk subjected to high pressure treatment (Table 2). The total vitamin C concentration in RHM samples was estimated at 34.4 mg per L of milk (Table 2). Pasteurization significantly reduced the content of vitamin C and AsA by 35% and 24%, respectively. Similar results were reported by Van Zoeren-Grobben *et al*. [1987] in whose study, LTLT pasteurization decreased vitamin C content of human milk by approximately 36%. In another study, vitamin C levels were reduced by around 20% and AsA concentrations decreased by around 16% when milk samples were heated at 62.5°C for 30 min [Moltó-Puigmarti *et al*., 2011].

A minor and statistically insignificant (approx. 6%) decrease in vitamin C levels was observed in milk subjected to high pressure of 193 MPa at a temperature of -20°C (Table 2). The above treatment led to a significant drop of more than 11% in AsA concentrations. The noted results suggest that AsA is a less stable form of vitamin C in human milk than DHsA. There are no published studies analyzing the influence of high pressure on the content of vitamin C and AsA in human milk under conditions similar to those applied in this experiment. Moltó-Puigmarti *et al.* [2011] did not report any changes in AsA levels and observed only a minor 4% drop in vitamin C concentrations in human milk subjected to pressures as high as 600 MPa at above-zero temperature.

In our study, the significant decrease in vitamin C and AsA content of milk processed by LTLT pasteurization, the drop in AsA concentrations in milk samples subjected to high pressure treatment and non-significant changes in TEAC values indicate that bioactive components of human milk other than vitamin C are characterized by a higher antioxidant activity and greater resistance to external stressors than vitamin C.

CONCLUSIONS

Our findings indicate that the content and composition of FAs, including LC-PUFAs, are not modified under exposure to high pressure at sub-zero temperature. LTLT pasteurization and the application of high pressure of 193 MPa at -20°C do not induce the formation of secondary oxidation products of LC-PUFAs, despite naturally high levels of those FAs in human milk.

LTLT pasteurization leads to a significant drop in the vitamin C content of human milk, whereas high pressure treatment at 193 MPa and -20°C does not exert a significant influence on vitamin C levels. Both treatments significantly reduce the AsA content of human milk, but the effect of pressurization is weaker. LTLT pasteurization causes a minor and statistically insignificant decrease in TEAC values, whereas milk samples subjected to high pressure retain their antioxidant properties. The influence of high pressure treatment on AsA levels and the absence of significant changes in TEAC values point to the relative stability of the remaining antioxidant components in human milk. Further research is needed to determine the effect of high pressure of approximately 200 MPa and sub-zero temperatures on other, mainly thermolabile, components of human milk, which are degraded by LTLT pasteurization.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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