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Analysis of Fatty Acid Binding Positions Lipid A Molecules by Periodate Oxidation and Mass Spectrometry

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Abstract

The immunological activity of LPS is closely related to the localization of fatty acids and the acyloxyacyl structures formed by them in lipid A molecules. MALDI-TOF mass spectrometry (MS) is commonly used to determine fatty acid localization by detecting oxonium ions derived from non-reducing end glucosamine by the split of glucosamine disaccharide of lipid A. However, an alternative method is required because the sensitivity in MS is reduced when the purity of lipid A preparation is not enough. To solve this problem, we developed the method of periodate oxidation. The glucosamine disaccharide of lipid A was split by sodium periodate after reduction with NaBH4 and the hemiacetal ring of reducing end glucosamine was opened. Through this oxidation, non-reducing end glucosamine with fatty acids bound to it remained and could be detected by MALDI-TOF MS. This method was applied to the wild-type lipid A, and the modified lipid A of Escherichia coli strains KGU0485 and KGU0496 constructed in the previous study by the introduction of Klebsiella acyltransferase gene. The results indicated that the method was useful for the determination of fatty acid localization in lipid A molecules.

Keywords

Lipid A
Fatty acid binding positions
Mass spectrometry
Periodate oxidation
Escherichia coli

1. Introduction

The importance of the chemical structure of lipid A, the active center of endotoxin (LPS), particularly the chain length and binding position of the fatty acid, for the immune activity of LPS was revealed in the 1980s by a group at Osaka University in their chemical synthesis studies [1]. This is because the specific shape of the hydrophobic region formed by fatty acids within the LPS molecule is required for specific association with the receptor TLR4. Determining the binding position of fatty acids within the lipid A molecule may therefore be crucial for predicting the activity of LPS. The hydrophobic region of *E. coli* lipid A, which is considered to have the strongest immune activity, is composed of acid-amide bonds at the C2 (amino group) and C3 (hydroxyl group) positions (expressed as C2, C3, C2’, and C3’ positions, respectively) of reducing-terminal and non-reducing terminal glucosamine, and

3-hydroxymyristic acid (3-OH-$C_{14:0}$), which is formed from two non-hydroxy fatty acids, namely lauric acid ($C_{12:0}$) and myristic acid ($C_{14:0}$), ester-bonded to the hydroxyl group of 3-OH-$C_{14:0}$, which is similarly bound to the non-reducing terminal glucosamine. We generated disruption mutants of the $C_{12:0}$ transferase gene or the $C_{14:0}$ transferase gene of *Escherichia coli*, which form such a branched-chain structure of fatty acids (acyloxyacyl structure) and attempted to produce lipids with the artificial structure A by introducing non-hydroxy fatty acid transferase genes of other bacterial species into these mutant strains [2,3]. In such studies, it is necessary to determine the exact binding position of the non-hydroxy fatty acid in the mutant lipid A obtained. In our recent studies, we have developed a method for analyzing the binding positions using periodate oxidation in addition to the conventional method. This method and its effectiveness are described in comparison with the conventional method.

2. Conventional method: detection of oxonium ions from non-reducing terminal glucosamine

To prepare lipid A from LPS, it is necessary to hydrolyze the linkages between the sugar backbone (glucosamine disaccharide) and the core sugar chain of lipid A with a relatively weak acid. This treatment releases part of the phosphate attached to the C1 position of the reducing terminal glucosamine. To simplify the hydrolysis products, we usually prepare and analyze dephosphorylated lipid A, in which the above phosphate is almost completely released, using the conditions 0.1 M HCl, 100°C, 30 minutes. Note that under these conditions, the phosphate group attached to the C4 position (C4' position) of the non-reducing terminal side glucosamine is not released.

Mass spectrometry of lipid A molecules with or without a phosphate group at the C1 position by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) shows that the bonds between the glucosamine disaccharides are broken and a cationic ion (oxonium ion) arising from the non-reducing terminal side of glucosamine can be detected in cation detection mode. This ion contains all the fatty acids bound to the non-reducing terminal side glucosamine, thus the type of fatty acid bound can be determined from the molecular weight. The flow of such an analysis is shown in **Figure 1**. It should be noted that which of the 3-OH-$C_{14:0}$ non-hydroxy fatty acids bonded to the C2' and C3' positions can be determined by hydrolysis treatment under weak alkaline conditions [4].

3. Periodate oxidation: cleavage of the reducing terminal glucosamine and detection of the resulting product

As mentioned above, the binding position of non-hydroxy fatty acids in lipid molecules can be determined by detecting oxonium ions, but the detection sensitivity is reduced by sample purity and foreign substances, and oxonium ions may not be clearly detected. Therefore, we devised an improved method to facilitate cleavage of the glucosamine disaccharide by reducing the C1 position of the reducing terminal glucosamine of dephosphorylated lipid A with sodium borohydride to open the ring to form a sugar alcohol, and we also investigated a more reliable method to localize the non-hydroxy fatty acids. The following is a brief description of the method.

As shown in **Figure 1**, the sugar alcohol obtained by the reduction of the C1 position gives rise to adjacent hydroxyl groups attached to the C4 and C5 positions, and it has long been known that the carbon bonds between such adjacent hydroxyl groups are cleaved by periodate oxidation. Although such chemical reactions are often accompanied by unexpected and non-specific reactions, the present study attempted to use this oxidation method to cleave the reducing terminal side of glucosamine. First, dephosphorylated lipid A was prepared from the LPS of *E. coli* strain KGU0107, which has wild-type lipid A. After ring-opening by reducing the C1 position, periodate oxidation (0.025 M $NaIO_4$, 4°C, 5 days) was performed. After the oxidation reaction, the

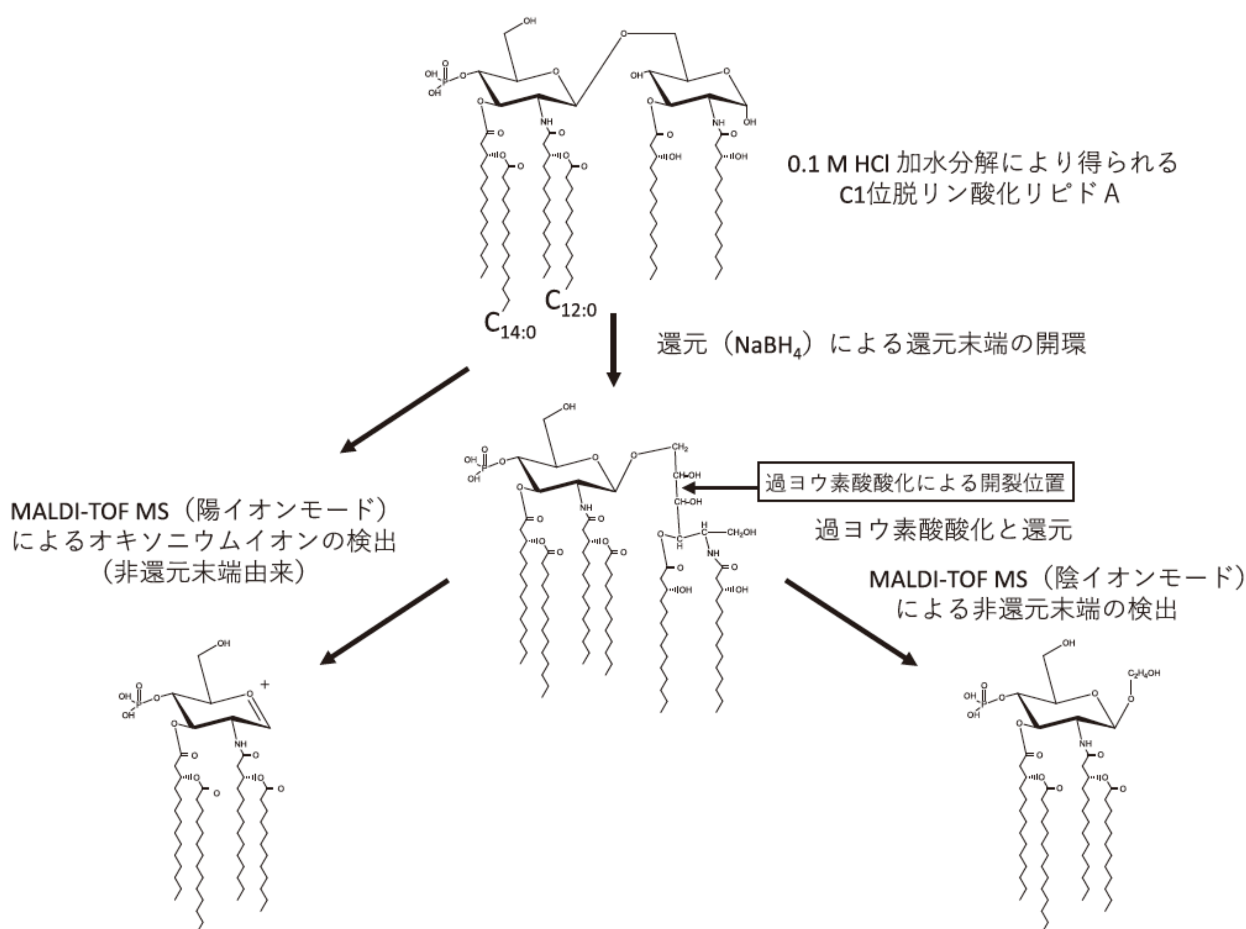

Figure 1. Schematic of the method for detection of non-reducing terminal side glucosamine fatty acid complexes

aldehyde group produced was reduced according to the usual method, and the product obtained was analyzed by MALDI-TOF MS (analyzed in anion detection mode), yielding the mass spectrum shown in **Figure 2**. In the spectrum, several peaks derived from dephosphorylated lipid A that had not undergone oxidation, such as *m/z* 1718.60 and *m/z* 1507.38, were observed, among which *m/z* 1147.12 and *m/z* 936.88 were presumed to be anions derived from non-reducing terminal glucosamine with structures 1 and 2 shown in the figure, respectively. These results suggest that the periodate oxidation method can be used to localize non-hydroxy fatty acids.

4. Application to the structural analysis of modified lipid A obtained by the introduction of fatty acid transferase genes

The above periodate oxidation method was applied to lipid A of strains KGU0485 and KGU0496, obtained by transfection of the *Klebsiella pneumoniae* $C_{14:0}$ transferase gene, to further validate its efficacy. Lipid A of these strains is presumed to have the structure shown in **Figure 3** [4,5].

First, the dephosphorylated lipid A of strain KGU0485 was reduced and periodate-oxidized, and then analyzed by mass spectrometry in the same way as above, yielding the spectrum shown in **Figure 4**. In this case, too, many peaks derived from lipid A that remained unoxidized were observed, but *m/z* 1175.10 was approximately 28 mass units (mu) (equivalent to two CH_2 molecules) larger than *m/z* 1147.12 in **Figure 2**, so it was assumed to be an ion with two molecules of $C_{14:0}$ as non-hydroxy fatty acids. In other words, both molecules of $C_{14:0}$ were found to exist on the non-reducing terminal side. On the other hand, the peak at *m/z* 964.90 was

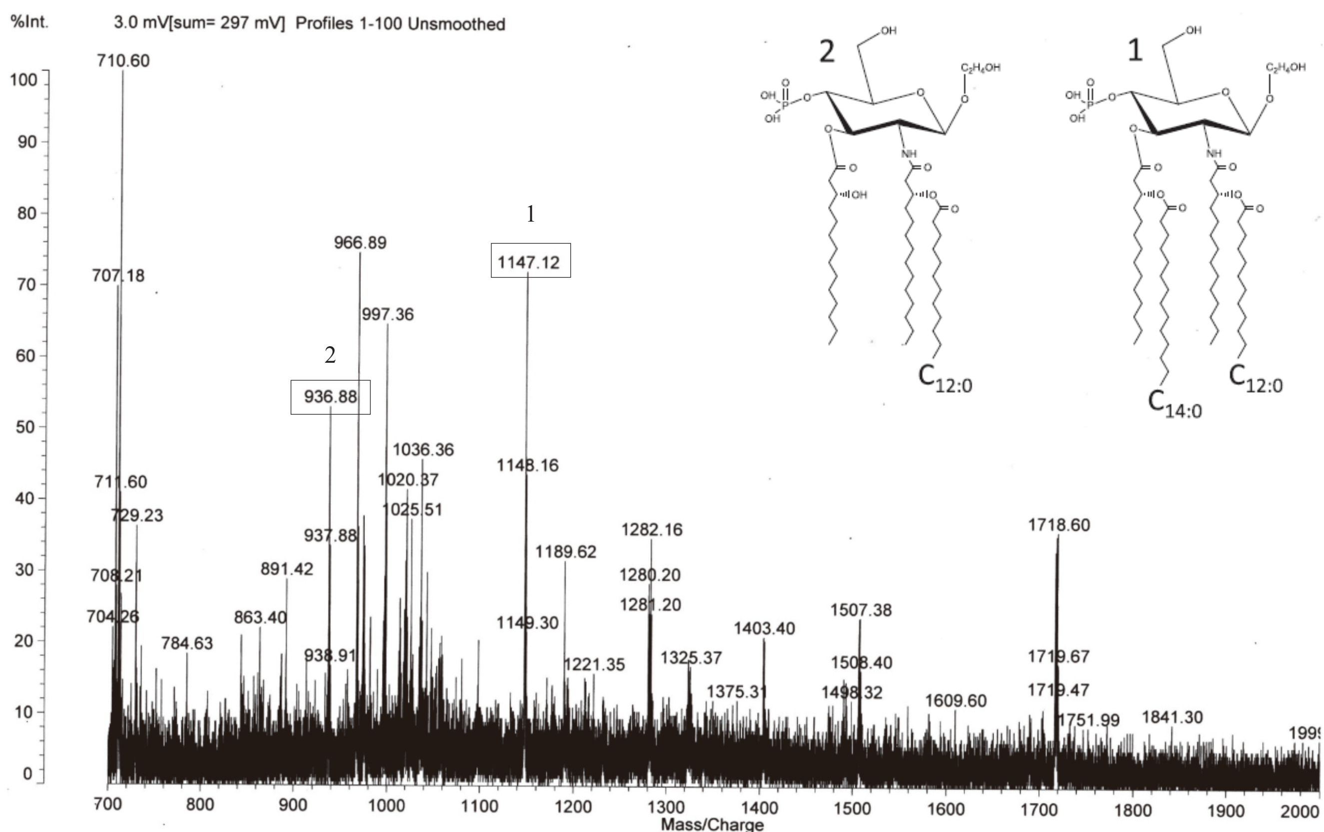

Figure 2. MALDI-TOF mass spectrum of periodate oxide obtained from strain KGU0107 (wild strain) lipid A

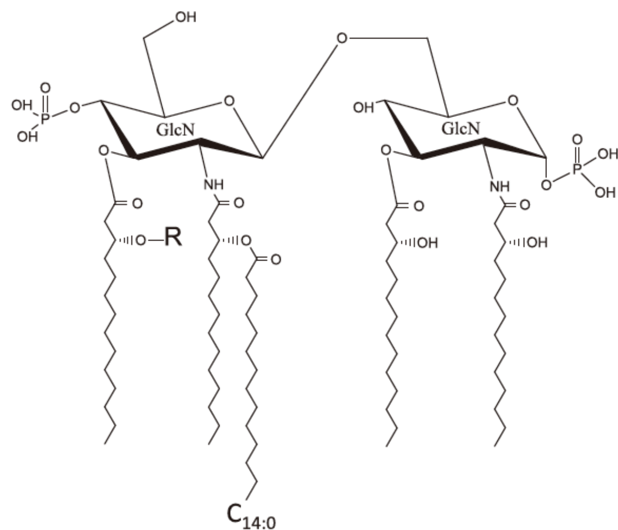

Figure 3. Predicted structure of KGU0485 and KGU0496 strain lipid A

approximately 210 mu smaller than *m/z* 1175.10 and was therefore considered to be a peak with one molecule of $C_{14:0}$ removed (calculated by subtracting H_2O from the molecular weight 228 of $C_{14:0}$).

Furthermore, when dephosphorylated lipid A from strain KGU0496 was analyzed after the same treatment, a distinct peak at *m/z* 964.81 was detected as the main peak, as shown in **Figure 5**. Therefore, this substance was also presumed to have a structure with one molecule of $C_{14:0}$, as shown in the figure. This proves that $C_{14:0}$ is present on the non-reducing terminal side of KGU0496 strain lipid A.

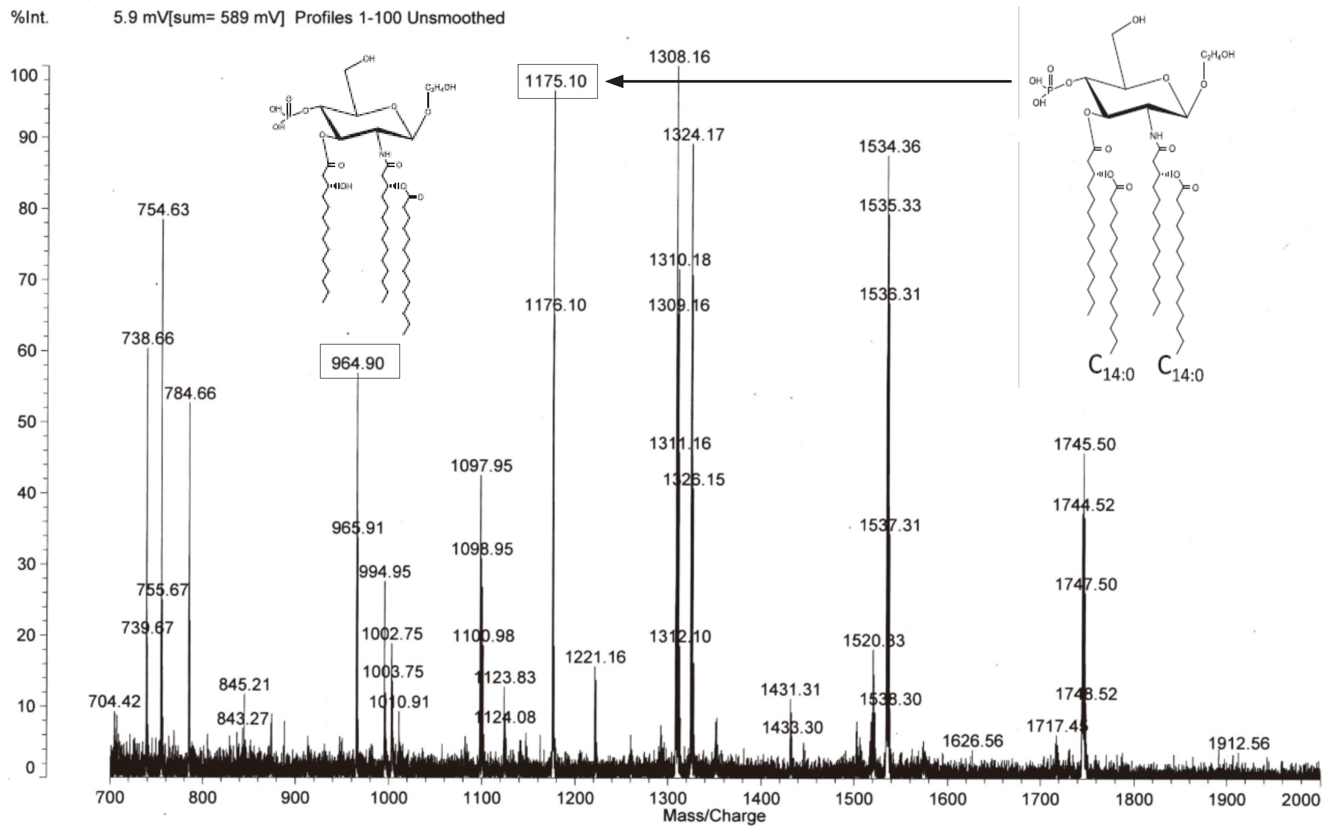

Figure 4. MALDI-TOF mass spectrum of periodate oxide obtained from KGU0485 strain lipid A

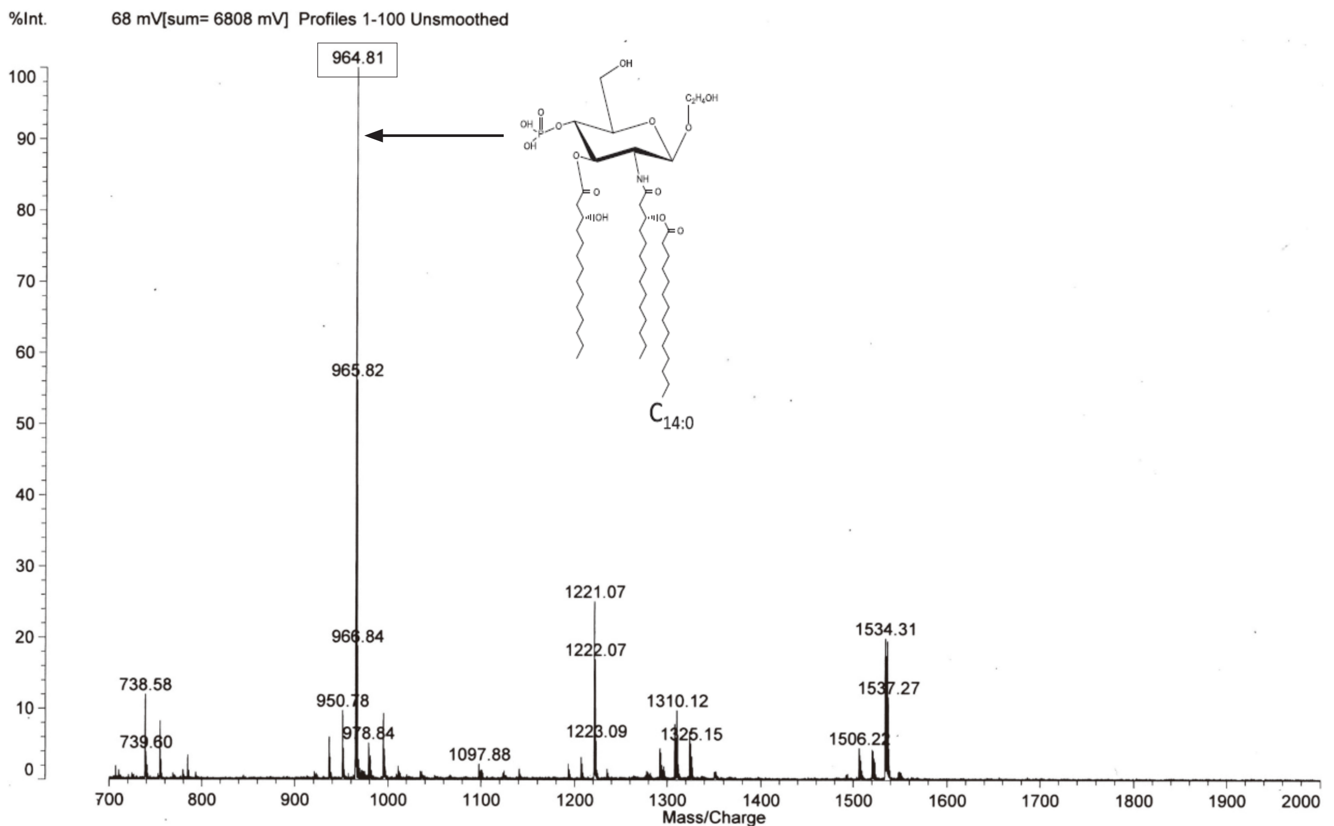

Figure 5. MALDI-TOF mass spectrum of periodate oxide obtained from KGU0496 strain lipid A

5. Conclusion

The method described in the first part of this review for detecting oxonium ions produced by the cleavage of glucosamine disaccharides is effective, but detection may be difficult due to the purity of the sample. In addition, MALDI-TOF MS may not be usable in some research environments. In comparison, compounds consisting of glucosamine and fatty acids from the non-reducing end of lipid A derived by periodate oxidation are expected to be detectable by liquid chromatography/mass spectrometry (LC-MS), a more common analytical tool. Thus, they may be more widely used. In addition, the structure shown in **Figure 5** is similar to a substance [6] that was previously studied as a compound exhibiting some of the activity of endotoxin. Therefore, the immune activity exhibited by this substance is also of interest. As seen in **Figures 4** and **5**, this sample contains lipid A molecules that have not been oxidized or cleaved by periodate, so it cannot be used for activity measurements in its present state, but further studies on purification methods and cytokine-producing activity of the purified product are necessary.

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Disclosure statement

The authors declare no conflict of interest.

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