Age-Related Changes in O-Acetylation of Sialic Acids Bound to N-Glycans of Male Rat Serum Glycoproteins and Influence of Dietary Intake on Their Changes

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ABSTRACT: O-Acetylation of sialic acids has been widely found in eukaryotic cells. Such modifications of sialic acids are tissue-specific and seem to be developmentally regulated. In this study, we performed comprehensive analysis of age-related changes in the serum N-glycans of male rats using capillary electrophoresis (CE) and investigated the changes in the O-acetylation of sialic acids bound to N-glycans with aging and different diets. The present method offered sufficient resolution to assess the degree of O-acetylation of the N-glycans and allowed for the determination of the age-related changes in O-acetylation of sialic acids. Using the CE-based method, we found that the relative abundance of disialo-biantennary N-glycans modified with 9-O-acetylated N-acetyleneuraminic acid (Neu5,9Ac) significantly increased with aging. In addition, the relative abundances of N-glycans with two Neu5,9Ac reversed to those of N-glycans with only Neu5Ac during 12 weeks. Next, we evaluated the influence of high-fat diet and food restriction on age-related changes in O-acetylation. Although the total amount of disialo-biantennary N-glycans increased with aging, age-related O-acetylation of sialic acids was suppressed by a high-fat diet. On the other hand, food restriction enhanced the O-acetylation of sialic acids, and the relative abundance of N-glycans with two Neu5,9Ac residues at 15 weeks of age was higher than that observed in the standard diet group. These findings suggest that the O-acetylation of sialic acids is closely related to changes in energy metabolisms such as glycolysis or fatty acid metabolism.

1. INTRODUCTION

Alterations in the physiological environment cause changes in the structure of oligosaccharides.1,2 Unlike protein sequences, which are primary gene products, N-glycan chain structures are not directly encoded in the genome. N-Glycans are synthesized in many different combinations by a variety of competing and sequentially acting glycosidases and glycosyltransferases without the use of a template. Small alterations in the environment can cause dramatic changes in the glycans produced by a particular cell; therefore, N-glycosylation could reflect the cell status. A spatial and temporal alteration in glycan expression implies that glycan structures are directly correlated with many physiological and pathological processes.3–6

Aging is a common physiological phenomenon occurring in any individual, and age-related changes in N-glycans during aging can often be observed as terminal monosaccharide modifications (i.e., galactosylation, fucosylation, and sialylation).7,8

Sialylation alters with development, growth, aging, and senescence. Altered sialylation in aged cells was initially reported in human and rat erythrocytes.7,9 The total sialic acid content of senescent red blood cells was 10–15% lower than that in younger red blood cells. The decreased sialic acid content from aging red blood cells is involved in the removal of senescent red blood cells during the blood circulation.9 On the other hand, it has been reported that the predominant linkage types of sialic acids change with aging. Tadokoro et al. reported that the α-2,6 sialylation of cell surface N-glycans on human diploid fibroblastic cells decreased markedly in aged cells when compared to young cells.10 Thus, the age-related changes in the terminal modification of N-glycans reflect the physiological status of cells, tissues, and bodies.

Sialic acids are commonly present in the outermost parts of complex-type N-glycans and mucin-type O-glycans in glycoproteins and glycolipids and play important roles in various biological events including fertilization, differentiation, tumor metastasis, virus infections, and modulation of intrinsic functions of glycoproteins (e.g., hormones and antibodies).11–16 O-Acetylations of sialic acids are also observed during tissue-specific and developmentally regulated expressions.17,18 In addition, sialic acids are often O-substituted with...
acetyl or sulfate groups at their 4-, 7-, 8-, and 9-positions, whereas the amino group at the 5-position is substituted with an N-acetyl or N-glycolyl group. For example, O-acetylation of sialic acids in erythrocytes blocks the binding of the influenza viruses A and B, while it is essential for influenza C binding.\textsuperscript{19,20} The amount of 9-O-acetylated GD3 ganglioside is closely correlated with aging, and the amount of 9-O-acetylation of sialic acids on N-glycans in serum glycoproteins. We found that the increase in the O-acetylation of sialic acids on N-glycans and allowed for the determination of O-acetylated NeuAc because the m/z values of Neu5Ac1Gal2Man3GlcNAc4-2AA and Neu5Ac2Gal2Man3GlcNAc4-2AA were observed between 17 and 24 min on the electropherogram (Supporting Information Figure S3). Minor peaks observed between 17 and 21 min that disappeared on digestion with jack bean \(\alpha\)-mannosidase, and a new single peak, which corresponds to \(\text{Man}\,1-4\text{GlcNAc}\,1-4\text{GlcNAc}\,2\text{AA}\), was observed at 16.5 min (Supporting Information Figure S3). These results indicate that these minor peaks correspond to high-mannose-type N-glycans (OS1-5).

The monosialo-fraction (1S) contained five high-mannose-type N-glycans (OS1-5) and six complex-type N-glycans (OS6-8, OS6f, OS7f, and OS8f). These N-glycans were observed between 17 and 24 min on the electropherogram (Supporting Information Figure S2). Minor peaks observed between 17 and 21 min that disappeared on digestion with jack bean \(\alpha\)-mannosidase, and a new single peak, which corresponds to \(\text{Man}\,1-4\text{GlcNAc}\,1-4\text{GlcNAc}\,2\text{AA}\), was observed at 16.5 min (Supporting Information Figure S3). These results indicate that these minor peaks correspond to high-mannose-type N-glycans (OS1-5).

Figure 1. Analysis of total N-glycans derived from male Wistar rat serum by CE. (a) Total N-glycans, (b) \(\alpha\)-L-fucosidase-treated total N-glycans, and (c) \(\alpha2,3,4,6,8,9\)-sialidase-treated total N-glycans: 0S1S, 1Sf, 2Sf, 3Sf, and 4Sf indicate asialo-monosialo-dissialo-trisialo- and tetrasialo-N-glycans, respectively, and 0Sf, 1Sf, 2Sf, 3Sf, and 4Sf indicate the presence of fucose residues. The monosaccharide compositions and tentative N-glycan structures in this region are summarized in Supporting Information Table S1 and Figure 2. 0Sf/0Sd indicate isomers of asialo-biantennary N-glycans (OS8), and 0Sf/0Sc indicate isomers of fucosylated asialo-biantennary N-glycans (OS8f).

Asialo- and high-mannose N-glycans were not retarded and observed between 4.0 and 5.0 min. Mono-, di-, tri-, and tetrasialo-N-glycans were observed in the order of the number of sialic acid residues. The asialo- and high-mannose fraction (OS) contained five high-mannose-type N-glycans (OS1-5) and six complex-type N-glycans (OS6-8, OS6f, OS7f, and OS8f). These N-glycans were observed between 17 and 24 min on the electropherogram (Supporting Information Figure S2). Minor peaks observed between 17 and 21 min that disappeared on digestion with jack bean \(\alpha\)-mannosidase, and a new single peak, which corresponds to \(\text{Man}\,1-4\text{GlcNAc}\,1-4\text{GlcNAc}\,2\text{AA}\), was observed at 16.5 min (Supporting Information Figure S3). These results indicate that these minor peaks correspond to high-mannose-type N-glycans (OS1-5).

The monosialo-fraction (1S) contained eight biantennary (1S1-4, 1S1f, 1S2f, 1S3f, and 1S4f) and two triantennary N-glycans (1S5 and 1S5f). Monosialo-glycan 1S3 (m/z 2052) was abundantly present, and its composition was determined to be Neu5Ac1Gal1Man3GlcNAc2-2AA. The molecular ion at m/z 2094 (1S4) was 42 mass units (corresponding to one acetyl group) higher than that of 1S3 and tentatively assigned to a monosialo-glycan with one O-acetylated Neu5Ac (Neu5,9Ac). N-glycans in the monosialo-fraction (1S) were observed as group g between 12 and 15 min in the electropherogram.

The most abundant disialo-fraction (2S) contained three disialo-biantennary and three fucosylated disialo-biantennary N-glycans. The molecular ions at m/z 2343.22 (2S1) and 2489.00 (2S1f) corresponded to glycans with the composition of \(\text{Neu5Ac}_1\text{Gal}_{1}\text{Man}_3\text{GlcNAc}_4\,2\text{AA}\) and \(\text{Neu5-}\text{Ac}_1\text{Gal}_{2}\text{Man}_3\text{GlcNAc}_4\,\text{Fuc}_2\,2\text{AA}\). The other four N-glycans were presumed to be disialo biantennary glycans modified with O-acetylated Neu5Ac because the m/z values of...
the molecular ions were 42 or 84 mass units (corresponding to 1−2 acetyl groups) higher than those of 2S1 or 2S1f. Disialo-glycans (2S) were observed between 10 and 12 min in the electropherogram. Although the groups 2S and 2Sf were composed of several peaks, it was considered that these peaks are because of the difference in the number of O-acetylated Neu5Ac residues.

The trisialo-fraction (3S) contained eight trisialo-biantennary and four trisialo-triantennary-N-glycans. The trisialo-glycans were observed between 9 and 10.5 min in the electropherogram. Two N-glycans (3S1 and 3Sf1) were abundantly present in this fraction, and the other six N-glycans were presumed to be trisialo-biantennary glycans having one or more O-acetylated Neu5Ac residues. The tetrasialo-fraction (4S) contained five tetrasialo-biantennary and three fucosylated tetrasialo-triantennary-N-glycans. The tetrasialo-glycans were observed between 8 and 9 min in the electropherogram. These characteristic tri- (3S1-4, 3S1f, 3S2f, 3S3f, and 3S4f) and tetrasialo-biantennary-glycans (4S1-5) have been reported to be present on the rat serum α1-acid glycoprotein and on bovine, pig, lamb, horse, and goat serum glycoproteins. It was suggested that two sialic acid residues

Figure 2. Serotonin-affinity chromatography of total N-glycans derived from male Wistar rat serum. 0S, 1S, 2S, 3S, and 4S mean asialo-, monosialo-, disialo-, trisialo-, and tetrasialo-N-glycans, respectively. The monosaccharide compositions of N-glycans observed in each fraction and their MS spectra and are shown in Supporting Information Table S1 and Figure S1. Symbols: gray diamond, 5'-N-acetyl-neuraminic acid (Neu5Ac); light gray diamond, 9-O-acetyl-5'-N-acetyl-neuraminic acid (Neu5,9Ac); gray circle, mannose (Man); black square, N-acetyl-glucosamine (GlcNAc); light gray circle, galactose (Gal); black triangle, fucose (Fuc); 2AA, 2-aminobenzoic acid.

Figure 3. Separation of disialo-biantennary glycans using an ODS column (a) and determination of the migration order of disialo-biantennary glycans (b). MS spectra of each fraction are shown in Supporting Information Figure S1. Analytical conditions (b): all conditions are the same as in Figure 1. Peak 2S1, 2S2, and 2S3 correspond to disialo-biantennary N-glycans having 0, 1, and 2 O-acetylated Neu5Ac, respectively. Peaks 2S1f, 2S2f, and 2S3f correspond to fucosylated N-glycans of 2S1, 2S2, and 2S3, respectively.
were attached to the galactose and GlcNAc residues on the nonreducing terminal lactosamine unit (Gal-GlcNAc-R).24,25

In summary, we found 47 N-glycans in male Wistar rat serum because the present method could be implemented without any loss of sialic acids through the experimental approach. However, the peak resolution was incomplete owing to the presence of O-acetylated Neu5Ac residues. The disialo fraction was further separated using an ODS column as the stationary phase.

As shown in Figure 3a, the disialo-biantennary glycans (2S fraction) fractionated by serotonin affinity chromatography were fractionated into three peaks by serotonin affinity chromatography. The most abundant peak A contained molecular ions at \( m/z \) 2343.35 and 2490.14 (Supporting Information Figure S4). Peaks a1 and a2 corresponded to 2S1 and 2S1f, respectively. Peak B contained two disialo-biantennary glycans with one O-acetylated Neu5Ac residue (2S2 and 2S2f), which were assigned as peak b1 and b2. The later eluted peak C corresponded to a disialo-biantennary glycan (2S3) and fucosylated disialo-biantennary glycan (2S3f). These disialo-N-glycans with two O-acetylated Neu5Ac were consistent with peaks b1 and b2. In the disialo fraction, monofucosylated disialo-biantennary glycans were consistent with the peaks a2, b2, and c2 as these peaks disappeared after digestion with \( \alpha-L \)-fucosidase. From these results, it was found that the migration order of sialo-glycans depends on the number of O-acetylated Neu5Ac residues.

N-Glycans with O-acetylated Neu5Ac residues were abundantly observed in male Wistar rat serum. The N-glycans on glycoproteins prepared from Wistar rat serum are quite complex even after the removal of sialyl residues. Although the applied analytical CE analytical technique was not sufficient to separate all N-glycan species, the approach can still be used as a comprehensive semiquantitative analysis for age-related changes in the O-acetylation of sialic acids bound to N-glycans. Based on the above-described structural information on serum N-glycans, we studied the relationship between the O-acetylation of sialic acids and aging and also investigated the effect of dietary intake on the O-acetylation process.

2.2. Alteration of the Amount of N-Glycans during Aging. First, we compared the mean total amounts of N-glycans in serum samples of three male Wistar rats of different ages (3−15 weeks) from the total fluorescence intensities of labeled N-glycans. For quantitative analysis, the 2AA-labeled N-glycan mixture containing internal standard (IS) was analyzed using a long capillary, and the peak areas corresponding to the N-glycans were corrected by the peak area of IS. The corrected peak areas were then applied to the calibration curve obtained from IS solutions, and the amounts of individual serum N-glycans were calculated. In this study, the amount of N-glycans in a single rat was calculated as the mean of triplicate measurements, and the amount of N-glycans times of 0S, and 0Sd did not change, whereas those of 0Sa and 0Sb shifted to earlier migration times (Supporting Information Figure S6). These results indicate that 0Sa, and 0Sd were asialo-N-glycans containing only type-II LacNAc units (Galβ1-4GlcNAc) at their nonreducing terminals. Observed peak fronting was due to the presence of a type-I LacNAc unit (Galβ1-3GlcNAc) at the nonreducing terminals of N-glycans. Although further studies are needed to determine the structures of these N-glycans, 0Sa, and 0Sb, were tentatively assigned to N-glycans having one or two type-I LacNAc units (Galβ1-3GlcNAc) at their nonreducing terminals of N-glycans.

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at each age was calculated as the mean of three independent rats. As shown in Figure 4a, the total amount of N-glycans increased from 12.2 ± 2.0 to 22.8 ± 2.1 nmol/mL serum during aging and markedly increased from 5 to 7 weeks.

The amounts of the tetrasialo-glycans 4S and 4Sf were 0.365–0.399 and 0.129–0.135 nmol/mL, respectively, and thus, the content of 4S was ca. approximately 2.5-fold higher than that of 4Sf at all ages (Figure 4b). The amounts of these tetrasialo-glycans changed slightly with aging, but the changes were not statistically significant (Figure 4c). The trisialo-glycans 3S and 3Sf were present at approximately 5-fold higher concentrations than the corresponding tetrasialo-glycans, but age-related changes in the trisialo-glycans were not observed (Figure 4c). The concentration of the most abundant disialo-glycans 2S and 2Sf at 3 weeks of age were 5.59 and 2.27 nmol/mL, respectively (Figure 4d). The disialo-glycans markedly increased with aging, and the amounts of 2S and 2Sf reached 13.00 and 4.13 nmol/mL, respectively (Figure 4d). The change in the level of disialo-glycans showed a trend similar to that of total N-glycans during aging. The increased amount of disialo-glycans (9.27 nmol sum of 2S and 2Sf) in 1 mL of serum was similar to that of the total N-glycans (10.20 nmol). These observations indicate that the age-related increase in the amount of total N-glycans was due to the increase in disialo-glycans. The amounts of monosialo- and asialo-/high-mannose glycans did also gradually increase with aging, but the changes were not significant (Figure 4e,f).

The structures of the disialo-glycans were very complex, and this complexity originated from the O-acetylation of Neu5Ac residues. The results are shown in Figure 5.

Disialo-glycans (2S) and monofucosylated disialo-glycans (2Sf) were resolved into four and three peaks, respectively. The structures of these glycans were determined in the same manner as indicated in Figure 3. Among the three disialo N-glycans, 2S2 exhibited two peaks around 31.5 min. It was assumed that these peaks represent positional isomers of O-acetylated Neu5Ac residues on different branches (e.g., Manα1-6 or Manα1-3 of complex-type N-glycans).

We compared the age-related changes in these six disialo-glycans and found that the amounts of all disialo-glycans increased in the serum with aging, with the increase being especially pronounced between 5 and 7 weeks. Among these six disialo-glycans, two disialo-glycans with O-acetylated Neu5Ac residues (2S2 and 2S3) markedly increased with aging (Figure 5b). The amounts of 2S2 and 2S3 at 3 weeks of age were 1.87 and 1.19 nmol/mL and increased to 4.55 and 3.68 nmol/mL at 15 weeks of age, respectively. Monofucosylated disialo-glycans with O-acetylated Neu5Ac residues (2S2f and 2S3f) also increased with aging, but the extent of the increase was less than those of 2S2 and 2S3. To evaluate the age-related changes in the O-acetylation of disialo-glycans, we compared the relative abundances of individual disialo-glycans with the abundance of the total disialo-glycans at different ages. The relative abundances of 2S1 and 2S3 at 3 weeks of age were 24.5 and 18.1% (Figure 5c); however, at 15 weeks, these relative abundances changed to 19.7 and 25.1%, respectively. Thus, the amounts of disialo glycans and the content of the O-acetylation of these disialo-glycans increased with aging.

Here, we focused on age-related changes in the O-acetylation of sialic acids and discussed the relationship between aging and the O-acetylation of disialo N-glycans. O-Acetylation of sialic acids bound to N-glycans may also be regulated by different core structures such as galactosyl linkage.
type and branching patterns. Accordingly, CE analysis with improved resolution will facilitate the understanding of O-acetylation regulation during aging in more detail.

### 2.3. Alteration of O-Acetylation of Sialic Acids on N-Glycans during Aging

Naturally occurring sialic acids can be O-acetylated at the 4-, 7-, 8-, or 9-hydroxyl positions. To determine the molecular species of sialic acids, we analyzed sialic acid analogues released by acid hydrolysis using high-performance liquid chromatography (HPLC) (Supporting Information Figure S7). Although sulfated Neu5Ac and 7,8,9-O-acetylated Neu5Ac (Neu5,7,8,9Ac3) were also reported as minor components in rat serum, these sialic acids were not detected in the present study. Neu5Gc and Neu5,7(8)Ac2 were only a few % content of total sialic acids for all weeks of age. Morimoto et al. evaluated the distribution of molecular species of sialic acids in rat tissues (7−9 weeks old) using HPLC coupled with electrospray ionization mass spectrometry. Most of the sialic acid species in rat serum are Neu5Ac (45.4%) and 9-O-acetylated Neu5Ac2 (40.1%).

| age       | relative abundance (%) to total sialic acida |
|-----------|---------------------------------------------|
| 3 weeks   | Neu5Ac 56.4 ± 2.72                           |
|           | Neu5Gc 2.28 ± 0.071                          |
|           | Neu5,7(8)Ac 4.95 ± 0.193                     |
|           | Neu5,9Ac 36.3 ± 1.13                         |
| 5 weeks   | Neu5Ac 53.9 ± 1.73                           |
|           | Neu5Gc 2.14 ± 0.102                          |
|           | Neu5,7(8)Ac 5.05 ± 0.106                     |
|           | Neu5,9Ac 38.6 ± 0.96                         |
| 6 weeks   | Neu5Ac 52.3 ± 1.09                           |
|           | Neu5Gc 2.26 ± 0.107                          |
|           | Neu5,7(8)Ac 5.07 ± 0.086                     |
|           | Neu5,9Ac 40.1 ± 1.07                         |
| 7 weeks   | Neu5Ac 51.3 ± 1.23                           |
|           | Neu5Gc 2.21 ± 0.130                          |
|           | Neu5,7(8)Ac 5.11 ± 0.169                     |
|           | Neu5,9Ac 41.0 ± 1.17                         |
| 10 weeks  | Neu5Ac 50.3 ± 1.29                           |
|           | Neu5Gc 2.64 ± 0.145                          |
|           | Neu5,7(8)Ac 5.16 ± 0.110                     |
|           | Neu5,9Ac 41.4 ± 0.88                         |
| 15 weeks  | Neu5Ac 46.9 ± 1.67                           |
|           | Neu5Gc 2.57 ± 0.134                          |
|           | Neu5,7(8)Ac 5.17 ± 0.099                     |
|           | Neu5,9Ac 44.4 ± 2.48                         |

aAbbreviation: Neu5Ac, N-acetyl-neuraminic acid; Neu5Gc, N-glycolyl-neuraminic acid; Neu5,9Ac, 9-O-acetyl-N-acetyl-neuraminic acid; Neu5,7(8)Ac, 7(8)-O-acetyl-N-acetyl-neuraminic acid. All data represent the mean ± SD relative abundances of Neu5A, Neu5Gc, Neu5,7(8)Ac, and Neu5,9Ac to total sialic acid (n = 3 independent experiments performed in triplicate).

Other species such as 7(8)-O-acetylated Neu5Ac2 (6.6%) and Neu5Gc (2.7%) are present less in rat serum. The relative abundances of the molecular species of sialic acids at 3−15 weeks of age are determined, as shown in Table 1.

The relative abundance of Neu5Ac was the highest at 3 weeks of age among all sialic acids, but it gradually decreased with aging. A small amount of Neu5Gc was observed at all ages, but the relative abundance did not change from 3−15 weeks of age. In contrast, the relative abundances of O-acetylated Neu5Ac (Neu5,9Ac2 and Neu5,7(8)Ac2) increased with aging. In detail, the relative abundances of Neu5,9Ac2 and Neu5,7(8)Ac2 were 36.3 and 4.95% at 3 weeks of age, and these increased to 44.4 and 5.17% at 15 weeks, respectively. Thus, it was concluded that the age-related alteration in the O-acetylation of Neu5Ac is due to the 9-O-acetylation of the Neu5Ac residue in glycoproteins.
Figure 7. Influence of FR on 9-O-acetylation of sialic acid on disialo-biantennary-glycans. (a) Electropherograms of disialo-biantennary-glycans in serum N-glycans prepared from male Wistar rats fed with SD every 2 days. Analytical conditions are the same as in Figure 5. Structures 2S1−2S3 and 2S1f−2S3f are shown in Figure 2. (b) It shows changes in the amount of disialo-biantennary-glycans (2S1−2S3 and 2S1f−2S3f). Symbols: filled circle, 2S1; filled square, 2S2; filled triangle, 2S3; open circle, 2S1f; open square, 2S2f; open triangle, 2S3f. All data represent the mean ± SD amounts of each disialo-biantennary N-glycan (2S1, 2S2, 2S3, 2S1f, 2S2f and 2S3f) in 1 mL sera (n = 3 independent experiments performed in triplicate). (c) It shows changes in relative abundance of three disialo-biantennary-glycans (2S1−2S3 and 2S1f−2S3f). All symbols are the same as in figure (b).

2.4. Influence of HF Diet and FR on the O-Acetylation of Sialic Acids in N-Glycans. Male Wistar rats (3 weeks of age: body weight, 49 ± 25 g) were fed with standard (SD) and HF diet for 12 weeks. The serum triglyceride (TG) level in rats fed with SD changed from 34.1 ± 11.9 to 77.9 ± 10.6 mg/dL (Supporting Information Figure S8). In contrast, the TG level in rats fed with HF diet markedly increased from 36.5 ± 13.1 to 107.5 ± 25.8 mg/dL, indicating that HF diet caused symptoms of lipid metabolism disorder in Wistar rats. The Wistar rats fed with HF diet showed a decrease in the O-acetylation of sialic acids.

Between 3 and 15 weeks of age, the total amount of disialo-glycans (the sum of 2S and 2Sf) increased from 6.28 to 12.82 nmol/mL serum with aging was similar to the increase observed in the SD group. This observation suggests that the production of glycoproteins in the liver and their secretion are not affected by the different diets. However, the age-related O-acetylation of Neu5Ac was markedly affected by the HF diet. The amounts of the disialo-glycans 2S2 and 2S3 increased only slightly with aging and their relative abundances at 15 weeks of age were 29.6 and 18.6%, respectively (Figure 6 b,c). In contrast, the amount and the relative abundance of 2S1 in the HF group increased markedly with aging (Figure 6c), and it was the most abundant glycan at 15 weeks of age (4.61 nmol/mL serum with aging was similar to the increase observed in the SD group. This observation suggests that the production of glycoproteins in the liver and their secretion are not affected by the different diets. However, the age-related O-acetylation of Neu5Ac was markedly affected by the HF diet. The amounts of the disialo-glycans 2S2 and 2S3 increased only slightly with aging and their relative abundances at 15 weeks of age were 29.6 and 18.6%, respectively (Figure 6 b,c). In contrast, the amount and the relative abundance of 2S1 in the HF group increased markedly with aging (Figure 6c), and it was the most abundant glycan at 15 weeks of age (4.61 nmol/mL, 35.2%). Furthermore, the elevated amount of 2S1 was maintained from 7 (3.41 nmol/mL, 32.2%) to 15 (4.51 nmol/mL, 35.2%) weeks. A decrease in the O-acetylation of Neu5Ac was also observed in the α1-6 fucosylated disialo-glycans 2S1f, 2S2f, and 2S3f (Figure 6c). Thus, HF diet decreased the age-related O-acetylation of Neu5Ac.

To investigate the influence of FR on age-related O-acetylation of Neu5Ac, we analyzed the disialo-glycan profiles in FR group in the same manner described above for the HF diet. The total amount of the disialo-glycans (the sum of 2Ss and 2Sfs) increased with aging and showed a similar age-related change as observed in the SD group. FR facilitated the age-related O-acetylation of sialic acids compared to that in the normal aging group (Wistar rats fed with SD), as shown in Figure 5. After FR for 2 weeks (5 weeks of age), the amount of disialo-glycans with two Neu5,9Ac (2S3) residues (1.95 nmol/mL, 19.3%) markedly increased compared to that in the normal aging group (1.52 nmol/mL, 23.8%). The higher content of disialo-glycans (2S3) was maintained from 7−15 weeks. In contrast, the amount of disialo-biantennary N-glycans (2S2 and 2S3) slightly increased with aging, but the relative abundances changed barely between 7 (30.6%) and 15 (32.5%) weeks. These results indicate that age-related O-acetylation of Neu5Ac on N-glycans is influenced by FR (Figure 7).

3. DISCUSSION

Modifications of sialic acids are tissue-specific and developmentally regulated in a variety of systems. O-Acetylation is one of the most frequently observed modifications of sialic acids in animals. Although rat serum glycoproteins carry O-acetylated sialic acids, it is not known whether the regulation of O-acetylation of serum glycoproteins is affected by physiological conditions such as aging or dietary habits.

O-Acetylation of sialic acids is regulated by at least three parameters; (i) balance between O-acetyltansferases and O-acetylestases together with substrate and cofactor availability, (ii) availability of the sialylated acceptor glycan, and (iii) availability of the acetyl group donor. Among them, O-acetyltansferases and O-acetylestases are key determinants in the O-acetylation of serum glycoproteins because both enzyme activities are higher in the rat liver. Furthermore, most serum glycoproteins, except γ-globulin fractions (i.e. IgG,
IgA, IgM), are synthesized in liver cells. Therefore, age-related changes in the O-acetylation of sialic acids might reflect changes in the activities of these enzymes because of the age-related physiological status of rat liver cells. On the other hand, the extent of the O-acetylation of sialic acids is not only determined by these two enzymes but also by availability of acetyl-CoA, which is an acetyl group donor, because availability of cellular acetyl-CoA depends on physiological metabolism conditions. At present, we do not have any evidence of the regulation of the O-acetylation of sialic acids, but further studies may lead to the discovery novel pathway and machinery involved in aging.

In the present study, we primarily focused on age-related changes in the N-glycosylation and O-acetylation of sialic acid on N-glycans. As described above, the O-acetylation of sialoglycans on serum glycoproteins seems to depend on balance between the activities of O-acetylation-related enzymes and the availability of acetyl-CoA in rat hepatic cells. Generally, the production of hepatic enzymes is easily affected by various physiological environments such as dietary habits and chronic stress. For example, the activity of the glycolytic enzyme glucokinase in hepatic cells was significantly reduced in Wistar rats fed with a low-protein diet compared with that in normally fed rats.30 Also, the serum levels of hepatocyte-specific enzymes, such as alkaline phosphatase and lactate dehydrogenase, are significantly increased in rats fed with a HF diet.31,32 In our studies, the total amount of N-glycans in the HF or SD group was similar, indicating that the HF diet did not influence the production of serum glycoproteins. In contrast, the O-acetylation of sialic acids markedly decreased in rats fed with the HF diet. Although the biochemical mechanisms underlying the changes in O-acetylation are not clear, we assume that the decrease in O-acetylation results from HF diet-associated changes in energy metabolism. HF diet induces hepatic enzymes such as acetyl-CoA carboxylase (ACC), which are involved in the regulation of fatty acid metabolism. ACC catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA; thus, an increase in ACC activity results in a decrease in available acetyl-CoA in hepatic cells. This finding supports our hypothesis that the decrease in O-acetylation is associated with HF diet-dependent changes in the availability of acetyl-CoA in hepatic cells. If the change in the O-acetylation of sialic acids is caused by the decrease in available acetyl-CoA, Neu5,9Ac2, carrying glycoproteins may be promising biomarkers for metabolic disorders.

In the present study, we also found that FR facilitates the O-acetylation of sialic acids in rats compared with that of SD group. FD has been shown to modulate various acetylation and deacetylation reactions of proteins. FR stimulates fatty acid oxidation in the liver, resulting in an increase in the [acetyl-CoA]/[CoA] ratio.33−35 FR-induced changes in the level of cellular acetyl-CoA may be an important determinant for the O-acetylation of sialic acids because acetyltransferases require acetyl-CoA as an acetyl group donor. On the other hand, FR also increases the cellular content of nicotinamide adenine dinucleotide (NAD).30 Intracellular NAD content affects various acetylation and deacetylation processes of proteins.37,38 FR-induced changes in cellular NAD are important determinants for protein acetylation. Although further studies are needed to determine the reasons for the FR-induced increase in the O-acetylation of sialic acids, FR-associated changes in O-acetylation may be influenced by complex mechanisms consisting of multiple factors that are involved in the acetyl-CoA and NAD metabolism pathways.

In these studies, we analyzed age-related changes in the O-acetylation of sialic acids in rat serum glycoproteins and the influence of different diets on these changes. We found that the O-acetylation of sialic acids is highly variable and sensitive indicator of changes in the internal physiological environment of hepatic cells. Although the investigations on age-related changes in the O-acetylation of sialic acids were restricted to male rats in this study, sex-dependent changes in glycosylation during aging may also occur because different types of sex hormones influence physiological activities such as metabolism, secretion, and digestion. Indeed, an N-glycan profiling study involving 265 human serum samples reported sex-dependent glycosylation changes (i.e., α1-3 fucosylation, bisecting GlcNAcation, and N-glycan branching).39 More extensive studies will require to reveal sex dependent O-acetylation changes and their underlying regulation mechanisms in rodents.

Interest in the O-acetylation of sialic acids is increasing because of their widespread occurrence and involvement in many cell biological and pathophysiological phenomena. For example, the 9-O-acetylation of sialic acids inhibits the recognition of nonacetylated sialic acids by hemagglutinins of influenza A and B viruses.19 Expression of O-acetylated sialic acids can also alter the action of bacterial sialidases.39−42 In rats, 9-O-acetylation is primarily found on Neu5Acα2-6Galβ1-4GlcNAc sequences of N-glycans, and 9-O-acetylation of the structure in CD22 on B lymphocytes regulates the differentiation of immature B lymphocytes.28,43 Thus, the 9-O-acetylation of sialic acids is capable of modulating a variety of biological processes, such as virus binding, bacterial sialidase action, tumor metastasis, and cell adhesion. An increase in O-acetylation during aging might indicate the acquisition of various biological functions required for normal aging because 9-O-acetylation is involved in host defense against pathogens, the development of the nervous system, and escape from unfavorable interactions, such as virus binding or tumor metastasis.

4. CONCLUSIONS

In this study, we performed a comprehensive analysis of age-related changes in the serum N-glycans of male Wistar rat and obtained the following three findings: (i) the total amount of disialo-biantennary N-glycans modified with Neu5,9Ac residues significantly increases with aging, (ii) the intake of HF diet decreased the O-acetylation of sialic acids in serum glycoproteins, and (iii) FR facilitated the O-acetylation of sialic acids. These findings suggest that the O-acetylation of sialic acids is closely related to changes in energy metabolism such as glycolysis or fatty acid metabolism, and the effects of aging and food intake on O-acetylation should be considered in future in vivo glycobiological studies.

5. EXPERIMENTAL SECTION

5.1. Reagents. N-Glycoamidase F (PNGaseF) was obtained from Roche Diagnostics (Minato-ku, Tokyo, Japan). The ultrafiltration membrane (MWCO: 3000 Da) was purchased from Sartorius Mechatronics (Shinagawa-ku, Tokyo, Japan). The fused silica capillary (100 μm i.d.) was obtained from Agilent Technologies (Santa Clara, CA, USA). COSMOSIL SC18-PAQ (4.6 mm I.D. × 150 mm) was
obtained from Nacalai Tesque (Nakagyo-ku, Kyoto, Japan). Other reagents and solvents were of the highest grade and were commercially available.

5.2. Animals. Male Wistar rats (clean grade; Japan SLC Inc., Shizuoka, Japan) were used throughout the study. All experimental procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals (KAPS-20-040, Kindai University). The animals were maintained at 22 °C under a 12:12 h light–dark cycle with free access to water. In the standard diet group (SD group), 3 week-old rats were fed a standard diet (Oriental Yeast Co., Tokyo, Japan) containing 23.6% proteins, 5.3% fat, and 71.0% carbohydrates for one to 12 weeks. The HF diet group (HF group) was maintained in a manner similar to a HF diet containing 18.6% proteins, 12.0% fat, and 69.3% carbohydrates (TestDiet, Richmond, IN, USA). Food consumption of the FR group was about 50–60% of that of the SD group. Three male rats of each age group were analyzed for age-related changes at different ages (3, 4, 5, 6, 7, 10, and 15 weeks), and blood samples were collected under anesthesia with intraperitoneal urethane at 1.5 g/kg. Sera were separated and stored at −80 °C until use.

5.3. Whole Glycoproteins from a Rat Serum Sample. A serum sample (100 μL) was filtered through an ultrafiltration membrane (MWCO: 3000 Da, 1.5 mL volume tube) to remove low-molecular weight materials and inorganic salts. After the retentate was recovered, the filter membrane was washed with 100 μL of distilled water, and the filtrate was filtered again. Both retentates were combined and lyophilized to dryness. The lyophilized material was dissolved in water (100 μL) and mixed with acetone solution (1.5 mL) containing 5% acetic acid, 5% triethylamine, and 5% water. After keeping the mixture at −20 °C for 1 h, it was centrifuged at 12,000g for 15 min, and the supernatant was discarded. The precipitate was washed with 75% ethanol (1 mL) three times and dried in vacuo.

5.4. N-Glycans from the Whole Glycoprotein Pool. The whole glycoprotein pool (100 μL as serum) obtained as described above was suspended in a solution (80 μL) containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. The mixture was kept in a boiling water bath for 10 min. After cooling, an aqueous solution of 10% NP-40 (48 μL) and 0.3 M phosphate buffer (pH 7.5, 58 μL) were added to the mixture. After addition of PNGase F (1 unit/μL), the mixture was incubated at 37 °C for 24 h. After keeping the mixture in the boiling water bath for 5 min, the mixture was mixed with 600 μL of 95% ethanol. After centrifugation at 12,000g for 15 min, the supernatant (N-glycans) was collected, lyophilized to dryness, and used for quantitative analysis.

5.5. Preparation of 2AA-Labeled N-Glycans. Released N-glycans (100 μL as serum) was dissolved in 2AA (200 μL solution), which was prepared by dissolution of 2AA (30 mg) and sodium cyanoborohydride (30 mg) in methanol (1 mL) containing 4% sodium acetate and 2% boric acid, and then, 5 μL of IS solution (NeoAcct2-3Galβ1-4Glc, 5 pmol) was added to the mixture. The mixture was maintained at 80 °C for 60 min. After the addition of distilled water (200 μL), the mixture was applied to a column of Sephadex LH-20 (1.0 cm i.d., length of 30 cm) equilibrated with 50% aqueous methanol. The earlier eluted fluorescent fractions (ex 340 nm, em 410 nm) were collected and evaporated to dryness. The lyophilized 2AA glycans were dissolved in 200 μL of Milli-Q water, and a portion was used for quantitative analysis.

5.6. Capillary Electrophoresis. CE was performed on an MDQ Glycoprotein System (Beckman Coulter) equipped with a helium–cadmium laser induced fluorescence detector (ex 325 nm and em. 405 nm) using a DB-1 capillary (100 μm i.d., effective length of 20 cm, total length of 30 cm, Agilent Technologies). The sample solution (5 μL) containing 2AA-labeled N-glycans (corresponding to 2.5 μL of serum) was introduced to the capillary by the pressure method (1.0 psi, 10 s). Separation was performed at 25 °C at an applied voltage of −25 kV (reverse polarity) using 50 mM Tris-borate buffer (pH 8.3) containing 10% polyethylene glycol 70,000 as the electrolyte. We also used a longer capillary (100 μm i.d., effective length of 60 cm, total length of 70 cm) to increase the resolution operated at −30 kV. Other analytical conditions were the same as described earlier when a 20 cm capillary was used. All electropherograms were analyzed using 32 Karat Software (Beckman Coulter), and peak areas were calculated by setting appropriate integration parameters. In the analysis of the alteration in the O-acetylation of sialic acids on N-glycans, automatic peak integration settings (i.e., integration range: 30–34 min, baseline type: vertical division, other settings: default) were used to calculate the peak areas of the six disialo-biantennary glycans. In analysis of the alteration in the amount of N-glycans, multiple peaks including the same number of sialic acid residues were treated as one peak group. During this quantitative analysis, the peak areas corresponding to N-glycans were corrected by the peak area of IS. The corrected peak areas were applied to the calibration curve obtained from IS solutions (n = 3, range from to 500 pmol/200 μL), and the amounts of individual N-glycans in serum were calculated.

5.7. Serotonin-Affinity Chromatography for Group Separation of N-Glycans Based on the Number of Sialic Acid Residues. The N-glycan pool (corresponding to 50 μL of serum) obtained from male rat serum as was separated based on the number of sialic acid residues using a serotonin-immobilized column; the procedure is described in the Supporting Information.44 Five fractions (0S, 1S, 2S, 3S, and 4S) were collected, lyophilized to dryness, and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (the procedure is described in the Supporting Information).

5.8. Separation of Disialo-Biantennary N-Glycans on a Silica-Based ODS Column. Separation was performed on an ODS column (Cosmosil SC18-PAQ, 4.6 mm i.d. × 150 mm length, Nakalai Tesque, Nakagyo-ku, Kyoto, Japan) using a Jasco HPLC apparatus equipped with a Jasco FP-920 fluorescence detector. Peaks were detected at 410 nm upon excitation at 340 nm. Elution was performed with a linear gradient using 15% acetonitrile in 0.025% acetic acid/1 mM tributylamine water as solvent A and 80% acetonitrile (in the same solution) as solvent B. The column was initially equilibrated and eluted with 3% solvent B for 2 min, and then, a linear gradient elution was performed with 60% of solvent B for 80 min. Peaks were collected and lyophilized to dryness. For the separation of disialo-biantennary N-glycans, the glycans fractionated by serotonin affinity chromatography were injected onto a C18 column. Three peaks (A, B, and C) were collected and reconstituted in 50 μL of distilled water. The solutions were then used for CE-based analysis and were subjected to MALDI-TOF MS analysis.

5.9. Analysis of Molecular Species of Sialic Acids in Total N-Glycans Derived from Male Wistar Rat Serum. Sialic acids at the nonreducing termini of N-glycans were
analyzed after hydrolysis with acetic acid followed by derivatization with 1,2-diamino-4,5-methylenedioxybenzene according to the method reported previously (the procedure is described in the Supporting Information)\textsuperscript{27,28}. The relative abundances of four sialic acid species (Neu5Gc, Neu5Ac, Neu5,7(8)Ac, and Neu5,9Ac) were calculated from their relative peak areas (Supporting Information Figure S7).

5.10. Data Collection and Analyses. A series of sample preparations (whole glycoproteins, whole N-glycans, and fluorescent-labeled N-glycans preparations) and CE-laser-induced-fluorescence analysis were performed in triplicate. The mean of the three measurements was used as the representative values for each individual serum sample. The means of the N-glycan amounts in each group at each age were calculated from three independent experiments. Data are expressed as mean $\pm$ SD from three independent experiments.

### ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00935.

Additional materials and methods; MALDI-TOF MS analysis of N-glycans fractionated by serotonin-affinity chromatography, peak assignment of N-glycans fractionated by serotonin affinity chromatography on a CE electropherogram, digestion of whole N-glycans with jack bean $\alpha$-mannosidase, MALDI-TOF MS analysis of disialo N-glycans fractionated by an ODS column, digestion of asialo N-glycans with $\alpha$-L-fucosidase, digestion of asialo N-glycans with $\beta$1-3 galactosidase, and changes in molecular species of sialic acids on N-glycans with aging, changes in serum TG levels with aging; and list of N-glycans observed in Wistar rat serum (PDF).

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**Notes**

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### REFERENCES

1. Kang J.-G.; Ko, J.-H.; Kim, Y.-S. Pros and cons of using aberrant glycosylation as companion biomarkers for therapeutics in cancer. BMB Rep. 2011, 44, 765–771.

2. Moremen, K. W.; Tiemeyer, M.; Nairn, A. V. Vertebrate protein glycosylation: diversity, synthesis and function. Nat. Rev. Mol. Cell Biol. 2012, 13, 448–462.

3. Knezević, A.; Gornik, O.; Polasek, O.; Pucic, M.; Redzic, I.; Novokmet, M.; Rudd, P. M.; Wright, A. E.; Campbell, H.; Rudan, I.; Lauc, G. Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma N-glycans. Glycobiology 2010, 20, 959–969.

4. Vanhooren, V.; Desmyter, L.; Liu, X.-E.; Cardelli, M.; Franceschi, C.; Federico, A.; Libert, C.; Laroy, W.; Dewaele, S.; Contreras, R.; Chen, C. N-glycanic changes in serum proteins during human aging. Rejuvenation Res. 2007, 10, 521–531a.

5. Haltiwanger, R. S.; Lowe, J. B. Role of glycosylation in development. Annu. Rev. Biochem. 2004, 73, 491–537.

6. Ohtsubo, K.; Marth, J. D. Glycosylation in cellular mechanisms of health and disease. Cell 2006, 126, 855–867.

7. Aminoff, D.; Bell, W. C.; VorderBruegge, W. G. Cell surface carbohydrate recognition and the viability of erythrocytes in circulation. Prog. Clin. Biol. Res. 1978, 23, 569–581.

8. Ding, N.; Nie, H.; Sun, X.; Sun, W.; Qu, Y.; Liu, X.; Yao, Y.; Liang, X.; Chen, C. C.; Li, Y. Human serum N-glycan profiles are age and sex dependent. Age Aging 2011, 40, 568–575.

9. Gutowski, K. A.; Hudson, J. L.; Aminoff, D. Flow cytometric analysis of human erythrocytes: I. Probed with lectins and immunoglobulins. Exp. Gerontol. 1991, 26, 315–326.

10. Lutz, H. U.; Fehr, J. Total sialic acid content of glycoporphins during senescence of human red blood cells. J. Biol. Chem. 1979, 254, 11177–11180.

11. Parekh, R.; Roitt, I.; Isenberg, D.; Dwek, R.; Rademacher, T. Age-related galactosylation of the N-linked oligosaccharides of human serum IgG. J. Exp. Med. 1988, 167, 1731–1736.

12. Tadokoro, T.; Yamamoto, K.; Kuwahara, I.; Fujisawa, H.; Ikikita, M.; Taniguchi, A.; Sato, T.; Furukawa, K. Preferential reduction of the $\alpha$-2-6-sialylation from cell surface N-glycans of human diploid fibroblastic cells by in vitro aging. Glycoconj J. 2006, 23, 443–452.

13. Tsuchiya, N.; Endo, T.; Matsuta, K.; Yoshinoya, S.; Takeuchi, F.; Nagano, Y.; Shiota, M.; Furukawa, K.; Kochibe, N.; Ito, K.; et al. Detection of glycosylation abnormality in rheumatoid IgG using N-acetylclycosamine-specific Pathyrella velutina lectin. J. Immunol. 1993, 151, 1137–1146.

14. Varki, A. Sialic acids as ligands in recognition phenomena. FASEB J. 1997, 11, 248–255.

15. Varki, A. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 1993, 3, 97–130.

16. Kageshita, T.; Hirai, S.; Hanai, N.; Ohta, S.; Ono, T. Association between sialyl Lewis(a) expression and tumor viability in human colon cancer. Glycoconjugate J. 2006, 23, 443–452.

17. Varki, A. Diversity in the sialic acids. Glycobiology 1992, 2, 25–40.

18. Klein, A.; Roussel, P. O-acetylation of sialic acids. Biochimie 1998, 80, 49–57.

19. Higa, H. H.; Rogers, G. N.; Paulson, J. C. Influenza virus hemagglutinins differentiate between receptor determinants bearing N-acetyl-N-glycolyl- and N,O-diacyteneuraminic acids. Virology 1985, 144, 279–282.

20. Herrler, G.; Rott, R.; Klenk, H. D.; Müller, H. P.; Shukla, A. K.; Schauer, R. The receptor-destructing enzyme of influenza C virus is neuraminidase-O-acetylerastase. EMBO J. 1985, 4, 1503–1506.

21. Sparrow, J. R.; Barnstable, C. J. A gradient molecule in developing rat retina: expression of 9-O-acetyl GD3 in relation to cell
type, developmental age, and GD3 ganglioside. *J. Neurosci. Res.* 1988, 21, 398–409.
(22) Varki, A.; Hooshmand, F.; Diaz, S.; Varki, N. M.; Hedrick, S. M. Developmental abnormalities in transgenic mice expressing a sialic acid-specific 9-O-acetyleterase. *Cell* 1991, 65, 65–74.
(23) Schauer, R. Achievements and challenges of sialic acid research. *Glycoconj. J.* 2000, 17, 485–499.
(24) Nakano, M.; Kakehi, K.; Tsai, M. H.; Lee, Y. C. Detailed structural features of glycan chains derived from 1-acid glycoproteins of several different animals: the presence of hypersialylated, O-acetylated sialic acids but not disialyl residues. *Glycobiology* 2004, 14, 431–441.
(25) Sumiyoshi, W.; Nakakita, S.-i.; Miyanishi, N.; Yamada, K.; Hasheira, K.; Nakakita, Y.; Hirabayashi, J. Hypersialylated type-1 lactosamine-containing N-glycans found in *Artiodactyla* sera are potential xenoantigens. *Glycobiology* 2012, 22, 1031–1041.
(26) Butor, C.; Diaz, S.; Varki, A. High level O-acetylation of sialic acids on N-linked oligosaccharides of rat liver membranes. Differential subcellular distribution of 7- and 9-O-acetyl groups and of enzymes involved in their regulation. *J. Biol. Chem.* 1993, 268, 10197–10206.
(27) Kabawata, A.; Morimoto, N.; Oda, Y.; Kinoshita, M.; Kuroda, R.; Kakehi, K. Determination of mucin in salivary glands using sialic acids as the marker by high-performance liquid chromatography with fluorometric detection. *Anal. Biochem.* 2000, 283, 119–121.
(28) Morimoto, N.; Nakano, M.; Kinoshita, M.; Kabawata, A.; Morita, M.; Oda, Y.; Kuroda, R.; Kakehi, K. Specific Distribution of Sialic Acids in Animal Tissues As Examined by LC–ESI-MS after Derivatization with 1,2-Diamino-4,5-Methylenedioxybenzene. *Anal. Chem.* 2001, 73, 5422–5428.
(29) Higa, H. H.; Manzi, A.; Varki, A. O-acetylation and de-O-acetylation of sialic acids. Purification, characterization, and properties of a glycosylated rat liver esterase specific for 9-O-acetylated sialic acids. *J. Biol. Chem.* 1989, 264, 19435–19442.
(30) Desai, M.; Byrne, C. D.; Meerman, K.; Martenz, N. D.; Bloom, S. R.; Hales, C. N. Regulation of hepatic enzymes and insulin levels in offspring of rat dams fed a reduced-protein diet. *Am. J. Physiol.* 1997, 273, G899–G904.
(31) Noeman, S. A.; Hamooda, H. E.; Baalash, A. A. Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. *Diabetol. Metab. Syndr.* 2011, 3, 17.
(32) Dhill, M.; Brahim, F.; Mnari, A.; Houas, Z.; Chargui, I.; Bchin, L.; Access, N.; Alsaïf, M. A.; Hammami, M. The intake of high fat diet with different trans fatty acid levels differentially induces oxidative stress and non alcoholic fatty liver disease (NAFLD) in rats. *Nutr. Metab.* 2011, 8, 65.
(33) Hagopian, K.; Ramsey, J. J.; Weindruch, R. Influence of age and caloric restriction on liver glycolytic enzyme activities and metabolite concentrations in mice. *Exp. Gerontol.* 2003, 38, 253–266.
(34) Wang, Z.; Masternak, M. M.; Al-Regaiey, K. A.; Bartke, A. Adipocytokines and the regulation of lipid metabolism in growth hormone transgenic and calorie-restricted mice. *Endocrinology* 2007, 148, 2845–2853.
(35) Hagopian, K.; Ramsey, J. J.; Weindruch, R. Caloric restriction increases gluconeogenic and transaminase enzyme activities in mouse liver. *Exp. Gerontol.* 2003, 38, 267–278.
(36) Chen, D.; Bruno, J.; Easlon, E.; Lin, S.-J.; Cheng, H.-L.; Alt, F. W.; Guarente, L. Tissue-specific regulation of SIRT1 by calorie restriction. *Genes Dev.* 2008, 22, 1753–1757.
(37) Lu, S.-P.; Lin, S. J. Regulation of yeast sirtuins by NAD(+) metabolism and calorie restriction. *Biochim. Biophys. Acta* 2009, 1804, 1567–1575.
(38) Kryrylenko, S.; Banahmad, A. Sirtuin family: a link to metabolic signaling and senescence. *Curr. Med. Chem.* 2010, 17, 2921–2932.
(39) Drzeniek, R. Substrate specificity of neuraminidases. *Histochem. J.* 1973, 5, 271–290.
(40) Corfield, A. P.; Veh, R. W.; Wember, M.; Michalski, J. C.; Schauer, R. The release of N-acetyl- and N-glycolloyl-neuraminic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases. *Biochem. J.* 1981, 197, 293–299.