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Effective detection and quantification of *dietetically* absorbed plant microRNAs in human plasma

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Abstract

The detection of exogenous plant microRNAs in human/animal plasma/sera lies at the foundation of exploring their cross-kingdom regulatory functions. It is necessary to establish a standard operation procedure to promote study in this nascent field. In this study, 18 plant miRNAs were assessed in watermelon juice and mixed fruits by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). C_T values, no-template controls and standard curves for each miRNA were used to evaluate the specificity and sensitivity of qRT-PCR and to obtain concentrations. Sixteen miRNAs were selected and measured in human plasma from volunteers after drinking juice. The C_T values of 6 plant miRNAs in human plasma fell outside the linear ranges of their standard curves. The remaining 10 miRNAs were present at high basal levels, and 6 of them showed a dynamic physiological pattern in plasma (absorption rates of 0.04% to 1.31%). Northern blotting was used to confirm the qRT-PCR results. Critical issues such as RNA extraction and internal controls were also addressed.

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Keywords: Extracellular microRNA; Exogenous microRNA; Plasma; Watermelon juice; Standard operation procedure

1. Introduction

Recent studies by our group and others have shown that microRNAs (miRNAs), a class of 19–24 nucleotide-long non-coding RNAs acting as post-transcriptional regulators of gene expression in eukaryotes [1–3], can circulate in the bloodstream and other bodily fluids in a stable, cell-free form [4–7]. Importantly, because extracellular miRNAs are aberrantly expressed in plasma, serum and other bodily fluids during the pathogenesis of many diseases, they may serve as promising noninvasive biomarkers to assess the pathological status of the body [4–7]. Further analysis suggested that these miRNAs exist in extracellular environment in a form that is enclosed in small membranous vesicles (e.g., exosomes, shedding vesicles and microvesicles) [8–12] or packaged with RNA-binding proteins (e.g., Argonaute 2) [13,14] or high-density lipoproteins [15]. According to current knowledge, extracellular miRNAs may be actively secreted by donor cells via membranous vesicles [8–12] or just merely by-products of cell death and released passively into the outer space after the cell membranes lose integrity [13]. Although these exciting discoveries open up the new research field of extracellular miRNAs, the measurement of extracellular miRNAs in circulation with adequate

sensitivity and precision comes with various challenges. (1) Because serum, plasma and other cell-free samples contain low amounts of extracellular miRNAs, an amplification step is required for most currently available quantification methods, such as the quantitative RT-PCR (qRT-PCR). Small variations in sample processing and enzymatic efficiency can result in large quantification errors. (2) Standard methods for cellular or tissue RNA extraction are inappropriate for extracellular RNA research. Because variations in RNA extraction can introduce potential bias, the development of a standard method for RNA extraction is critical for evaluating extracellular miRNAs. (3) To date, the systematic selection and validation of suitable internal controls for the quantification of extracellular miRNAs remains rather empirical, and no commonly accepted internal controls are yet available. These factors make it difficult to accurately detect the level of extracellular miRNAs in circulation.

In the process of obtaining a genome-wide expression profile of extracellular miRNAs in human serum using Solexa sequencing, we found not only endogenous human miRNAs but also a considerable quantity of plant miRNAs. This discovery led to our further identification of the cross-kingdom regulation of plant MIR168a on the expression of mouse liver low-density lipoprotein receptor adapter protein 1 (LDLRAP1) [16]. The presence of exogenous plant miRNAs and their kinetics in human and animal plasma/serum have been rigorously studied by various investigators ever since this discovery. Although some follow-up studies have detected plant miRNAs in human plasma [17], others have obtained contradictory

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results [18–20]. For example, Witwer et al. selectively measured MIR160, MIR156, MIR166, MIR167, MIR168 and MIR172 in the plasma of pigtailed macaques that received a plant miRNA-enriched substance by qRT-PCR and did not observe any consistent and specific amplification of plant miRNAs [18]. Snow et al. tested three miRNAs, MIR156a, MIR159a and MIR169a, and did not detect them in the plasma of healthy subjects after the intake of miRNA-containing fruits [19]. These inconsistencies are likely derived from the common technical issues in the extracellular miRNA research field, including the low concentration of miRNAs, the lack of a standard method for RNA extraction and the lack of proper internal controls. There are some additional factors, particularly the selection of plant miRNAs, that need to be considered in detecting exogenous plant miRNAs in human and animal plasma/serum. According to our model, plant miRNAs in food were absorbed by mouse epithelial cells in the gastrointestinal (GI) tract, re-packaged into MVs and released into circulation [16]. Our previous results showed that among the several thousands of plant miRNAs detected in plants, only approximately one dozen plant miRNAs were readily detected in human or animal plasma [16], suggesting that the intake of plant miRNAs through the GI tract is likely selective. It cannot be assumed that a miRNA enriched in plants will be present at a high level in plasma; therefore, randomly detecting plant miRNAs with high abundance in humans or animals can easily fail. Furthermore, due to the nature of miRNAs, non-specific signals can be high for certain miRNAs. Therefore, no-template controls must be assayed to reduce the non-specificity of qRT-PCR.

In this study, we strove to solve the controversial issues of detecting human and animal uptake of plant miRNAs from the diet. To establish a standard operation procedure (SOP) for measuring plant miRNAs in humans and animals, we systematically measured the kinetics of fruit-derived plant miRNAs in the peripheral blood of healthy volunteers after they drank watermelon juice or ate fruits. We also compared the methods used by our group and other groups in assaying plant miRNAs in mammals and addressed additional technical problems that may have previously caused inconsistent results in measuring exogenous plant miRNAs.

2. Results

2.1. Detection of plant miRNAs in watermelon juice and mixed fruits by a qRT-PCR assay

Twelve-milliliter volumes of watermelon juice were used for RNA extraction, and 18 plant miRNAs (MIR156a, MIR157a, MIR158a,

MIR159a, MIR160a, MIR162a, MIR163a, MIR166a, MIR167a, MIR168a, MIR169a, MIR170a, MIR172a, MIR390a, MIR395a, MIR528, MIR824 and MIR894) were assessed by a TaqMan probe-based qRT-PCR assay. To determine the specificity of each primer set, no-template controls for each miRNA were assessed simultaneously. All miRNAs except MIR170a and MIR395a were consistently and efficiently amplified. The C_T values of these miRNAs ranged from 14.58 to 27.98, while the C_T values of the respective no-template controls ranged from 25.84 to 36.80. The ΔC_T values ranged from -4.45 to -12.02 (Table 1). The C_T values of MIR170a and MIR395a could not be distinguished from the C_T values of the corresponding no-template controls (Table 1), suggesting that these miRNAs cannot be detected by the qRT-PCR assay. The levels of these 18 plant miRNAs in mixed fruits were also measured, and the data showed that all the miRNAs except MIR170a and MIR395a were detected (Table 1). Based on this analysis, we selected the remaining 16 plant miRNAs as candidates and quantified their levels in human plasma after the volunteers drank watermelon juice.

To obtain the concentrations of the 16 plant miRNAs in watermelon juice and mixed fruits, each synthetic single-stranded miRNA was serially diluted and assessed using the qRT-PCR assay to generate a standard curve. Each miRNA had a Pearson correlation coefficient (R) >0.99 (Supplementary Fig. 1). The no-template controls amplified at a much higher cycle range than the miRNAs. For each miRNA, the linear range of the C_T value (e.g., 7.00–28.02 for MIR156a) and the dynamic quantification range of the expression level (e.g., 0.01 amol to 100 fmol for MIR156a) are listed in Table 2. The concentrations of the plant miRNAs in watermelon juice and mixed fruits were calculated based on reference to the standard curve (Table 2). Both watermelon juice and mixed fruits carried some plant miRNAs. While MIR156a, MIR162a, MIR167a, MIR528, MIR166a and MIR159a were more enriched in watermelon juice, the mixed fruits contained relatively higher levels of MIR157a, MIR168a, MIR172a, MIR390a, MIR894, MIR158a, MIR160a, MIR163a, MIR169a and MIR824.

2.2. Detection of plant miRNAs in plasma after the oral administration of watermelon juice

Watermelon juice was orally administered to nine healthy volunteers following overnight fasting. Blood was drawn at 0, 0.5, 1, 3, 6 and 9 h after drinking. One hundred microliters of plasma was used for RNA extraction and qRT-PCR analysis. In total, 10 plant miRNAs (MIR156a, MIR157a, MIR162a, MIR167a, MIR168a, MIR172a, MIR390a, MIR528, MIR894 and MIR166a) were consistently and

Table 1
The raw C_T values of plant miRNAs in watermelon juice and mixed fruits detected by qRT-PCR.

| Plant miRNA | Sequence | C_T value in watermelon juice | C_T value in fruit mixture | C_T value in no-template control |
|-------------|--------------------------|---------------------------------|------------------------------|------------------------------------|
| ath-MIR156a | UGACAGAAGAGAGUGAGCAC | 20.08±0.24 | 23.68±0.41 | 28.82±0.01 |
| ath-MIR157a | UUGACAGAAGAUAGAGAGCAC | 18.75±0.52 | 13.85±0.49 | 30.77±0.06 |
| ath-MIR162a | UCGAUAAACCUUCGCAUCCAG | 21.96±0.66 | 26.03±0.41 | 31.63±0.27 |
| ath-MIR167a | UGAAGCUGCCAGCAUGAUCUA | 25.03±0.24 | 31.47±0.03 | 36.14±0.01 |
| ath-MIR168a | UCGCUUGGUGCAGGUCGGGAA | 23.05±0.26 | 25.29±0.41 | 31.76±0.04 |
| ath-MIR172a | AGAAUCUUGAUGAUGCUGCAU | 22.29±0.30 | 22.20±0.38 | 31.29±0.08 |
| ath-MIR390a | AAGCUCAGGAGGGAUAGCGCC | 22.61±0.18 | 20.12±0.34 | 31.70±0.80 |
| osa-MIR528 | UGGAAGGGGCAUGCAGAGGAG | 20.23±0.27 | 25.63±0.37 | 28.48±0.02 |
| ppt-MIR894 | CGUUUCACGUCGGGUUACC | 14.58±0.87 | 13.70±0.34 | 33.25±0.21 |
| ath-MIR166a | UCGACCAGGCUUCAUUCCCC | 25.66±0.29 | 28.20±0.41 | 36.80±0.02 |
| ath-MIR158a | UCCCAAUUGAACAAGCA | 23.91±0.08 | 20.17±0.02 | 33.57±0.03 |
| ath-MIR159a | UUUGGAUUGAAGGGAGCUCUA | 21.90±0.02 | 26.15±0.04 | 33.54±0.14 |
| ath-MIR160a | UGCCUGGCUCGCCUGAUGCCA | 24.94±0.27 | 21.86±0.37 | 29.39±0.07 |
| ath-MIR163a | UUGAAGAGGACUUGGAACUUCGAU | 27.98±0.55 | 28.80±0.50 | 34.63±0.08 |
| ath-MIR169a | CAGCCAAGGAUGACUUGCCGA | 25.70±0.35 | 25.89±0.46 | 31.82±0.09 |
| ath-MIR824 | UAGACCAUUUGUGAGAAGGGA | 20.22±0.25 | 21.87±0.41 | 25.84±0.44 |
| ath-MIR170a | UGAUUGAGCCGUGUCAUAUUC | 28.02±0.08 | 26.53±0.47 | 28.02±0.04 |
| ath-MIR395a | CUGAAGUGUUUGGGGAACUC | 29.03±0.01 | 28.18±0.06 | 29.72±0.66 |

Table 2
The dynamic quantification range for plant miRNA measurement and the concentration of plant miRNAs in watermelon juice and fruits.

| Plant miRNA | Linear range of C _T values | Dynamic quantification range of expression levels | Concentration in watermelon juice (pM) | Concentration in fruit mixture (pmol/kg) |
|-------------|---------------------------------------|---|--|--|
| ath-MIR156a | [7.00–28.02] | [0.01 amol–100 fmol] | 310.82 | 204.89 |
| ath-MIR157a | [8.34–30.02] | [0.01 amol–10 fmol] | 1207.03 | 11315.29 |
| ath-MIR162a | [6.46–28.47] | [0.001 amol–100 fmol] | 20.54 | 6.59 |
| ath-MIR167a | [10.71–32.18] | [0.001 amol–10 fmol] | 25.33 | 2.06 |
| ath-MIR168a | [10.33–29.35] | [0.001 amol–10 fmol] | 34.84 | 56.33 |
| ath-MIR172a | [8.05–29.84] | [0.01 amol–100 fmol] | 191.5 | 496.06 |
| ath-MIR390a | [6.60–27.85] | [0.001 amol–100 fmol] | 41.83 | 65.18 |
| osa-MIR528 | [10.23–26.88] | [0.1 amol–10 fmol] | 1048.01 | 298.11 |
| ppt-MIR894 | [10.21–32.51] | [0.01 amol–10 fmol] | 39846.54 | 55192.46 |
| ath-MIR166a | [11.17–30.47] | [0.01 amol–10 fmol] | 30.42 | 0.01 |
| ath-MIR158a | [8.82–29.43] | [0.01 amol–10 fmol] | 39.88 | 4782.72 |
| ath-MIR159a | [7.96–27.01] | [0.1 amol–100 fmol] | 347.49 | 169.8 |
| ath-MIR160a | [9.23–26.57] | [1 amol–100 fmol] | 373.54 | 1850.46 |
| ath-MIR163a | [11.23–30.95] | [0.01 amol–10 fmol] | 6.66 | 37.49 |
| ath-MIR169a | [8.48–28.59] | [0.01 amol–100 fmol] | 12.99 | 112.72 |
| ath-MIR824 | [6.40–22.83] | [0.1 amol–100 fmol] | 107.52 | 275.1 |
| ath-MIR170a | [8.47–23.93] | [1 amol–100 fmol] | UD | UD |
| ath-MIR395a | [7.39–23.85] | [0.1 amol–100 fmol] | UD | UD |

specifically amplified. Supplementary Fig. 2 shows the individual C_T-vs.-time curves for these 10 miRNAs. The C_T values of MIR156a, MIR157a, MIR162a, MIR168a, MIR172a, MIR390a, MIR528 and MIR894 were generally within the linear range at all time points. The C_T values of MIR167a were outside the linear range at the 0 h time point but entered the linear range after administration. The C_T values of MIR166a were in the linear range but showed no changes during the experimental period. The C_T values of the remaining 6 miRNAs (MIR158a, MIR159a, MIR160a, MIR163a, MIR169a and MIR824) were outside the linear range (Supplementary Fig. 3). As negative controls, the C_T values of MIR170a and MIR395a were also outside the linear range (Supplementary Fig. 3).

The concentrations of the 10 detectable plant miRNAs in the plasma were calculated by referring to the standard curves. MIR528 showed the highest basal level in plasma (8289.02 fM) and reached its peak (16141.78 fM) three hours after juice administration (Fig. 1A). MIR156a, MIR162a, MIR168a, MIR172a and MIR390a had basal concentrations of 317.10, 33.45, 28.49, 82.24 and 10.38 fM, respectively, and peaked at 1 or 3 h after juice administration at 933.12, 118.57, 56.62, 162.32 and 18.41 fM, respectively (Fig. 1A and B). The MIR166a level did not change after drinking (Fig. 1B). Unexpectedly, the levels of MIR157a and MIR894 increased consistently in the plasma after drinking the juice, and their concentrations changed from 33.64 and 576.37 fM, respectively, at the basal level to 115.88 and 1753.44 fM, respectively, at the end point (Fig. 1A and B). Notably, the basal level of MIR167a in human plasma was negligible but significantly increased after drinking, reaching 797.47 fM at the end point (Fig. 1C). The basal level, peak concentration and fold change of each plant miRNA after juice administration are listed in Table 3. Six plant miRNAs (MIR156a, MIR162a, MIR168a, MIR172a, MIR390a and MIR528) showed typical kinetic absorption curves and they were used to calculate the total amount of plant miRNA absorbed by the body. Based on our calculations of the concentrations of plant miRNAs in watermelon juice and the areas under the plasma concentration-vs.-time curves (AUCs) of the plant miRNAs, we determined the total uptake amount, total absorption amount and absorption rate of each plant miRNA. The absorption rates ranged from 0.04% to 1.31% (Table 3).

As controls, the levels of four endogenous miRNAs (miR-16, miR-21, miR-25 and miR-92a) were examined during this process. The endogenous miRNAs did not change consistently in the volunteers' plasma after the administration of watermelon juice (Fig. 1D and

Supplementary Fig. 3). This quantification also revealed that some plant miRNAs are present in the plasma in a similar concentration range to endogenous miRNAs (e.g., MIR894 vs. miR-16, MIR156a vs. miR-92a and MIR172a vs. miR-25). In agreement with our previous study showing that the plant miRNAs detected in circulation are mainly encapsulated in microvesicles (MVs), MIR156a, MIR162a and MIR168a were primarily detected in the MV fraction of human plasma and were present in nearly negligible amounts in the MV-free fraction (Supplementary Fig. 4). The endogenous miRNAs (miR-16, miR-21 and miR-223) were detected in both the MV and the MV-free fractions of human plasma (Supplementary Fig. 4).

2.3. Validation of plant miRNAs in watermelon juice or plasma by northern blotting

Northern blotting was used to confirm the qRT-PCR results. In this assay, digoxigenin (DIG)-labeled oligonucleotide probes containing locked nucleic acids (LNA) allow the sensitive and highly specific detection of mature miRNAs [21]. First, synthetic single-stranded miRNAs (MIR156a, MIR162a and MIR168a) were serially diluted and assessed using northern blotting. The results showed that plant miRNA could not be detected at levels less than 100 amol (Supplementary Fig. 5). Then five plant miRNAs (MIR156a, MIR162a, MIR168a, MIR157a and MIR166a) in watermelon juice were assessed. Fig. 2A shows that plant miRNAs were readily detected in as little as 100 µl of watermelon juice. Furthermore, the five plant miRNAs and an endogenous miRNA (miR-16) in the plasma were assessed. Fig. 2B shows that plant miRNAs were readily detected in 4 ml of human plasma and that the levels of MIR156a, MIR162a and MIR168a increased after consumption, reached their peak levels and then dropped; MIR157a consistently increased in the plasma after juice administration; MIR166a was present in human plasma at a low level (approximately 10 fM×4 ml=40 amol) and therefore undetectable by northern blotting; and, as a control, miR-16 remained unchanged over time.

2.4. Detection of plant miRNAs in the plasma after eating mixed fruits

The kinetics of the plasma levels of plant miRNAs after the healthy volunteers ate mixed fruits was measured. The experimental procedure was the same as the administration of watermelon. Supplementary Fig. 6 shows the concentration-vs.-time curves for plant miRNAs in the plasma after the administration of mixed fruits. As shown, the kinetics of the plant miRNAs after fruit administration appeared similar to the kinetics after juice administration. The levels of MIR156a, MIR157a, MIR162a, MIR172a, MIR390a and MIR168a in the plasma increased dramatically following fruit administration.

2.5. Analyses of RNA extraction methods, absolute plasma miRNA concentrations and internal controls

The recovery rate of RNA after extraction by phenol/chloroform-based (phenol/chloroform and Trizol LS) or column-based (MirVana) methods was assessed by qRT-PCR analysis with a readout of C_T values and reproducibility. Samples extracted by the phenol/chloroform method showed the lowest C_T values, and samples extracted by the MirVana method showed the highest C_T values (Fig. 3A–C and Supplementary Fig. 7). When comparing the reproducibility based on the standard error of the mean (SEM) of C_T values, the best method was the phenol/chloroform method, and the worst was the MirVana method (Fig. 3A–C). When RNA was extracted using the phenol/chloroform and Trizol LS methods, alterations in the plant miRNAs in the plasma were detected regardless of whether they were present in the plasma at high or low levels (Fig. 3A–C and Supplementary Fig. 7). In contrast, alterations in plant miRNAs were detected for abundant miRNAs (e.g., MIR528) but not for miRNAs present at relatively low

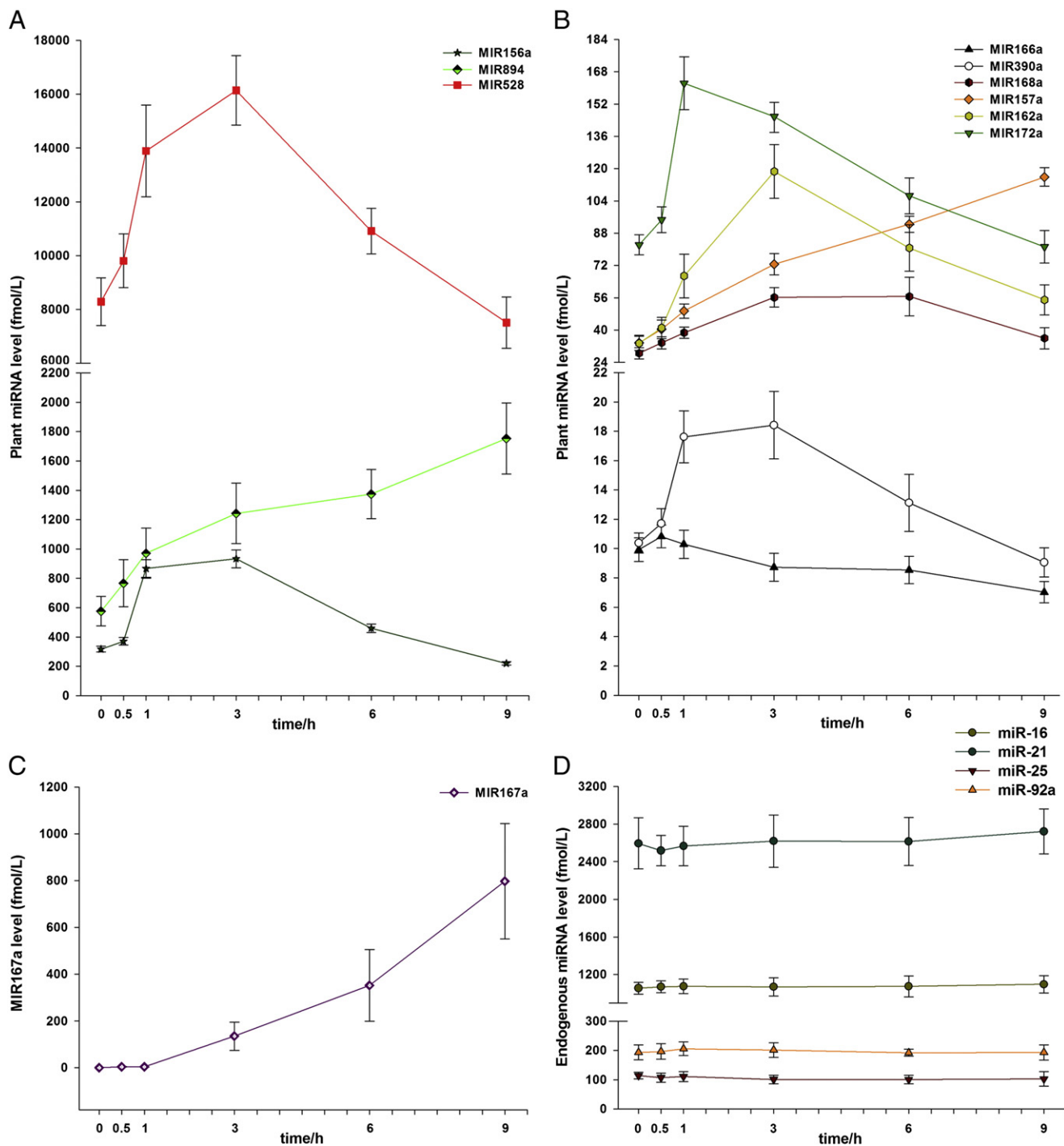


Fig. 1. Kinetics of the appearance of plant miRNAs in the plasma after the administration of watermelon juice. (A) Mean concentration-*vs.*-time curves for MIR156a, MIR528 and MIR894 in the plasma of healthy subjects after a single administration of watermelon juice. (B) Mean concentration-*vs.*-time curves for MIR157a, MIR162a, MIR166a, MIR168a, MIR172a and MIR390a in the plasma of healthy subjects after a single administration of watermelon juice. (C) Mean concentration-*vs.*-time curves for MIR167a in the plasma of healthy subjects after a single administration of watermelon juice. (D) Mean concentration-*vs.*-time curves for miR-16, miR-21, miR-223 and let-7d/g/i in the plasma of healthy subjects after a single administration of watermelon juice. The values are presented as the mean \pm S.E.

levels (e.g., MIR156a and MIR168a) in RNA extracted using the MirVana method (Fig. 3A–C and Supplementary Fig. 7).

However, even the most effective RNA isolation protocols cannot retain the entire small RNA fraction, and therefore, the calculated plant miRNA concentration in the plasma may be underestimated. To estimate the absolute levels of plant miRNAs in watermelon juice and plasma, synthetic MIR156a at a known concentration was added to

watermelon juice or plasma, and the RNA was then extracted and measured by a qRT-PCR assay. Fig. 3D shows the new standard curve generated using extracted watermelon juice RNA at a concentration range of five orders of magnitude. The linear range of the C_T values shifted from 9.01–28.22 to 12.97–20.17 (Fig. 3D). Based on the new standard curve, the absolute concentration of MIR156a in the watermelon juice was approximately six times the previously

Table 3
The basal level, peak level and absorption rate of plant miRNAs in plasma following watermelon juice administration.

| Plant miRNA | Basal level in plasma (fM) | Peak level in plasma (fM) | Total uptake amount (pmol) | Total absorption amount (pmol) | Absorption rate (%) |
|-------------|----------------------------|---------------------------|----------------------------|--------------------------------|---------------------|
| ath-MIR156a | 317.1 | 933.12 | 777.05 | 2.59 | 0.33 |
| ath-MIR162a | 33.45 | 118.57 | 51.35 | 0.43 | 0.84 |
| ath-MIR168a | 28.49 | 56.62 | 87.1 | 0.18 | 0.21 |
| ath-MIR172a | 82.24 | 162.32 | 478.75 | 0.34 | 0.07 |
| ath-MIR390a | 10.38 | 18.41 | 104.58 | 0.04 | 0.04 |
| osa-MIR528 | 8289.02 | 16141.78 | 2620.02 | 34.37 | 1.31 |

calculated concentration. Fig. 3E shows the new standard curve generated using extracted plasma RNA at a concentration range of five orders of magnitude. The linear range of the C_T values shifted from 8.82–28.02 to 12.27–25.44 (Fig. 3E), and the absolute concentration of MIR156a in the plasma after juice administration was approximately four times the previously calculated concentration (Fig. 3F).

Using a correct control is critical for assessing alterations in extracellular RNA in circulation. Previous studies have suggested that endogenous miR-16 and let-7d/g/i were suitable internal controls to correct for variations in RNA recovery and amplification efficiency [22–24], and that the *Caenorhabditis elegans* miRNA cel-miR-39 was ideal spike-in control [25,26]. Thus, we selected miR-16 and let-7d/g/i as internal control and cel-miR-39 as spike-in control. Fig. 3G showed that the C_T values of miR-16, let-7d/g/i and cel-miR-39 did not change after juice administration. When assessing the levels of MIR156a and MIR168a in human plasma after juice administration, there were no differences between the groups with and without normalization by miR-16, let-7d/g/i or cel-miR-39 (Fig. 3H). After normalization with miR-16 or let-7d/g/i, significant inductions of MIR156a and MIR168a were observed in the plasma of mice after feeding with fresh rice compared with feeding a chow diet; however, no difference was observed when the mouse plasma levels of MIR156a and MIR168a were not normalized (Fig. 3I).

3. Discussion

Accumulating evidence has demonstrated that miRNAs, once thought to be unstable molecules, are stable and can be horizontally transferred across species and kingdoms [13,27]. However, to accurately detect the plasma or serum levels of extracellular miRNAs, particularly exogenous plant miRNAs, various technical issues must still be addressed. In this study, employing both qRT-PCR and northern blotting assays, we systematically characterized the kinetics of plant miRNAs in human

plasma after healthy volunteers drank watermelon juice. To develop a SOP for assessing the level of dietetically absorbed plant miRNAs in human or animal plasma, we systematically analyzed the factors, such as the selection of plant miRNAs, the RNA extraction method and the proper internal controls, that interfere with miRNA detection results.

The choice of plant miRNAs to be detected by qRT-PCR in human and animal plasma/serum can affect the outcome. The selection of plant miRNAs involves at least two main factors: a) the concentration of the miRNA in plants and the potential selective intake of the plant miRNA by human and animal GI tracts. To obtain the concentration of a plant miRNA, a specific standard curve and a no-template control are both required. Due to the limitation of TaqMan probe-based qRT-PCR, non-specific signals (e.g., C_T value of no-template control) are often obtained. For example, we found that among the 18 plant miRNAs tested, MIR170a and MIR395a generated C_T values that could not be distinguished from the values of the corresponding no-template controls, indicating that these two miRNAs have high background noise and cannot be correctly measured by qRT-PCR analysis. A specific standard curve for individual plant miRNAs in plasma is key not only for calculating the concentration of plant miRNAs but also for showing the effective quantification range. If the level of a miRNA is outside the linear working range of its standard curve, that miRNA is not suitable for detection by a qRT-PCR assay. In this study, the C_T values of 6 plant miRNAs (MIR158a, MIR159a, MIR160a, MIR163a, MIR169a and MIR824) in plasma were outside the dynamic quantification range, indicating that they had undetectable or unreliable levels in plasma and could not be accurately assessed. This result agrees with the previous studies in which the authors reported negative results in detecting MIR160a, MIR159a and MIR169a in human and animal plasma by qRT-PCR after the administration of a plant-based food [18,19].

Another factor that may affect the selection of plant miRNAs is the potential selective intake of the miRNA by humans and animals. Our previous study suggested that food-derived plant miRNAs are probably absorbed by the epithelial cells of the GI tract and then re-packaged into MVs and released into the circulation [16]. The genome-wide plant miRNA expression profile detected by Solexa sequencing identified more than 1000 miRNAs in rice; however, fewer than 20 plant miRNAs were readily detected in animal plasma [16]. This result suggests that food-derived miRNAs are selectively taken up by the animal GI tract. Therefore, miRNAs with high concentrations in plants do not necessarily result in high levels in human or animal plasma. Along this line, the failure to detect six plant miRNAs (MIR158a, MIR159a, MIR160a, MIR163a, MIR169a and MIR824) in human plasma, although they are all highly abundant in watermelon juice, may be due to their poor absorption by the human GI tract. To find the

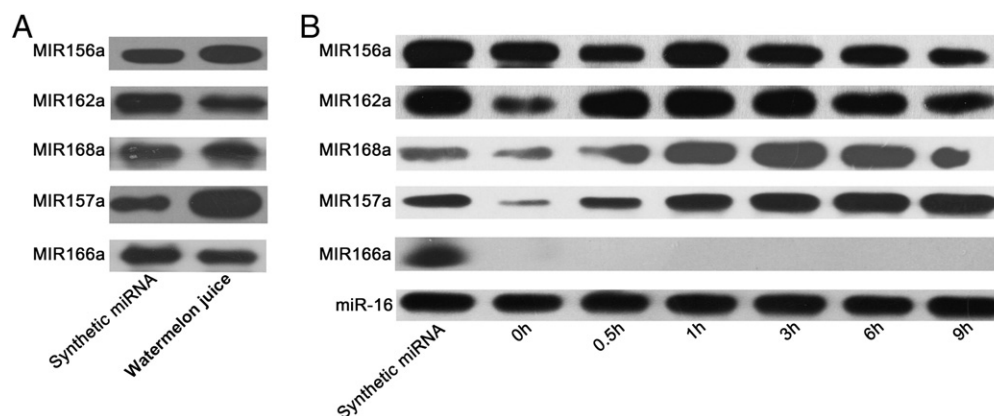


Fig. 2. Northern blot analysis to detect the accumulation of plant miRNAs in watermelon juice and in the plasma after the administration of watermelon juice.

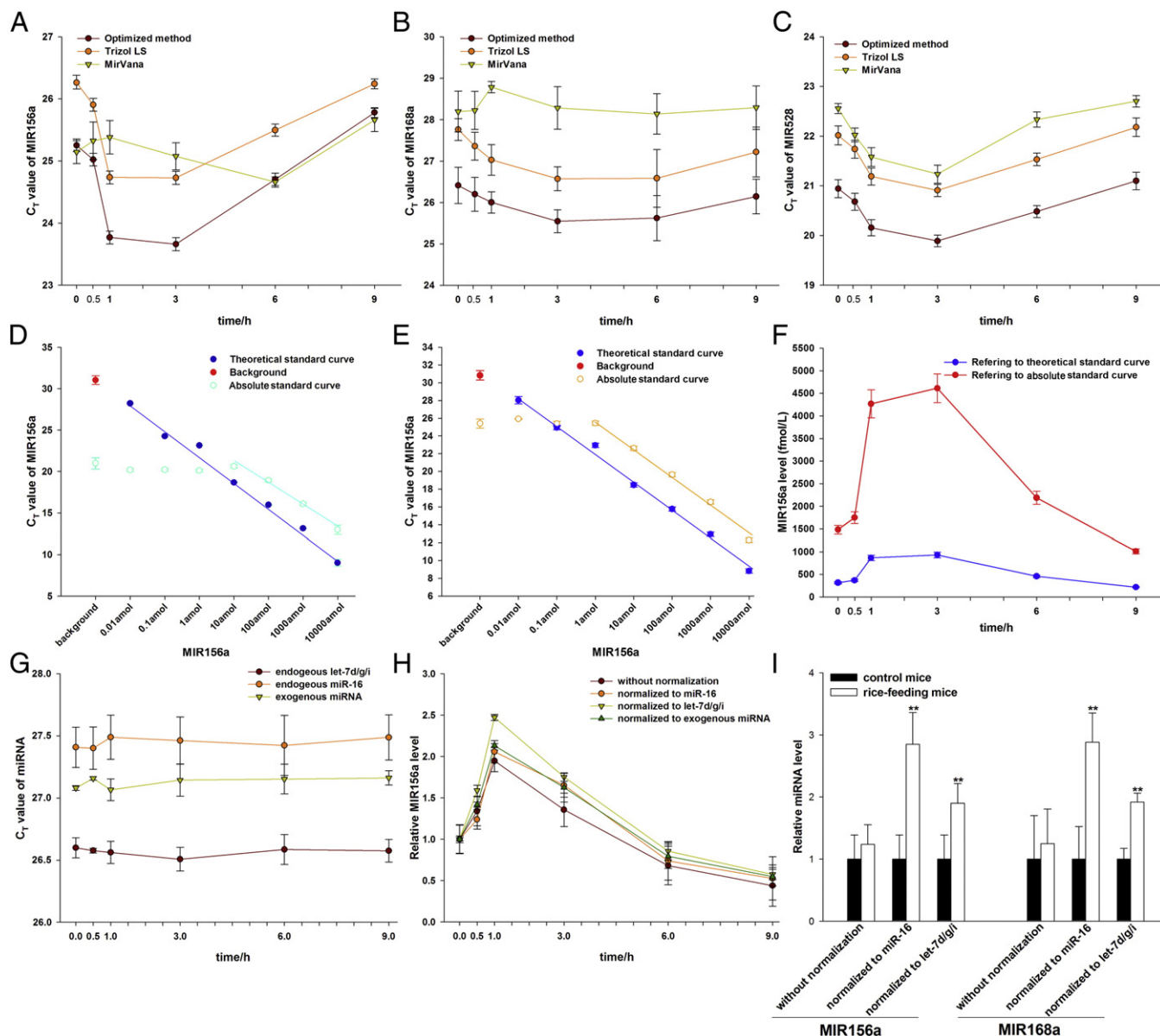


Fig. 3. Analyses of RNA extraction methods, absolute plasma miRNA concentrations and internal controls. (A–C) Selection of a suitable method for plant miRNA extraction from plasma. Mean C_T -vs.-time curves for MIR156a, MIR168a and MIR528 in plasma samples extracted by different RNA extraction methods. RNA in the plasma samples of healthy subjects after the administration of watermelon juice was isolated by our optimized phenol/chloroform extraction method, the Trizol LS method and the MirVana method, and the levels of MIR156a, MIR168a and MIR528 were quantified using a qRT-PCR assay. (D–F) Evaluation of the absolute concentrations of plant miRNAs in watermelon juice and plasma. (D) Theoretical and absolute standard curves of MIR156a were generated by adding synthetic MIR156a at a known concentration to water or watermelon juice before RNA isolation and then quantifying using a qRT-PCR assay. The resulting C_T values were plotted against the input MIR156 amounts. (E) Theoretical and absolute standard curves of MIR156a were generated by adding synthetic MIR156a at a known concentration to water or plasma before RNA isolation and then quantifying using a qRT-PCR assay. The resulting C_T values were plotted against the input MIR156 amounts. (F) Absolute concentration of MIR156a in human plasma by referring to the absolute standard curve. (G–I) Effect of normalization on the levels of plant miRNAs in plasma. (G) Mean C_T -vs.-time curves for miR-16, let-7d/g/i and cel-miR-39 in plasma of healthy subjects after a single administration of watermelon juice. The values are the means \pm SE. (H) The levels of MIR156a in the plasma of healthy subjects after a single administration of watermelon juice were measured by a qRT-PCR assay and were directly calculated or normalized to miR-16, let-7d/g/i or cel-miR-39. The relative levels were calculated using the $2^{-\Delta\Delta CT}$ method and are presented as mean fold changes \pm standard errors. (I) The levels of MIR156a in the plasma of mice after feeding with a control diet or rice were measured by qRT-PCR and either directly calculated or normalized to miR-16 or let-7d/g/i. The relative levels were calculated using the $2^{-\Delta\Delta CT}$ method and are presented as mean fold changes \pm standard errors. Significance was assessed by t-tests.

“right” plant miRNAs in plasma to be detected by qRT-PCR, a screening process by Solexa sequencing or a miRNA microarray may be required.

MVs are small vesicles that are shed from donor cells to mediate intercellular communication by transporting bioactive molecules, including miRNAs, between cells. Unlike circulating endogenous miRNAs, which were present both in MVs and the MV-free fractions [14], plant miRNAs were found to be largely encapsulated in MVs and nearly undetectable in the MV-free fraction of human plasma. Furthermore, we showed that some plant miRNAs are present in the

plasma in a similar concentration range to endogenous miRNAs. Thus, like endogenous miRNAs, plant miRNAs are present in human plasma in a form that can be easily delivered to other cells and at a concentration that is biologically relevant. Therefore, plant miRNAs in circulation fulfill the requirements for a miRNA to exert a physiological function and may be as important as endogenous miRNAs.

The process of extraction of RNA from the plasma or serum greatly affects the outcome of the quantitative detection of plant miRNAs. We found that column-based RNA extraction, a highly sensitive method

recently used by many investigators in RNA extraction from plasma samples, cannot thoroughly break MVs and separate binding proteins from miRNAs. This technique would result in substantial losses of small RNAs. Instead, our results showed that the phenol/chloroform extraction method had the best RNA recovery rate and reproducibility for the quantitative detection of plant miRNAs in human plasma. Therefore, a suitable RNA isolation method must be selected to accurately detect plant miRNAs in human and animal plasma.

The accurate normalization of the detected RNA levels is another critical issue in accurately measuring extracellular miRNAs. Due to the high sensitivity of qRT-PCR, any small variations in the experimental process can be amplified and contribute to quantification errors. The development of an internal control is indispensable for evaluating extracellular miRNAs. The miRNAs in tissues and cells are normalized by U6 [28–30]; however, this small RNA is degraded in the circulatory system [28–30]. Recently, it has been reported that miR-16 and let-7d/g/i can serve as internal controls for the detection of circulating miRNAs [22–24]. Here, we found that miR-16, let-7d/g/i and other endogenous miRNAs (miR-21, miR-25 and miR-92a) all maintained constant levels during the experimental period. We therefore used miR-16 and let-7d/g/i as internal controls, and our results suggested that a suitable internal control should be considered when plant miRNAs are detected in plasma. As shown in the mouse experiments, given the low volume of plasma obtained from a mouse and the relatively low levels of MIR156a and MIR168a in the plasma, normalization by miR-16 and let-7d/g/i is necessary. Indeed, after normalization, a significant induction of MIR156a and MIR168a was observed in the plasma of mice after feeding with fresh rice compared with feeding a chow diet, whereas there was no difference without normalization. Therefore, the internal control is an essential factor and should be carefully considered when a low concentration of plant miRNAs is detected in a small volume of plasma.

In this study, after addressing various factors that may interfere with the outcome of the quantitative detection of plant miRNAs in human plasma, we effectively and quantitatively measured the kinetics of plant miRNAs in plasma after healthy volunteers drank watermelon juice. The qRT-PCR results were further validated by a northern blot analysis using LNA-modified oligonucleotide probes. This study clearly demonstrates that plant miRNAs in human plasma can be efficiently detected and reliably compared by qRT-PCR and also provides a SOP for measuring plant miRNAs in human and animal plasma. By serving as a guideline, this SOP may clarify the controversial issues in the quantification of exogenous plant miRNAs in plasma, serum and other bodily fluids of humans and animals.

4. Materials and methods

4.1. Subjects

Nine male volunteers were recruited into this study. All the subjects were healthy and had no history of disease. The subject characteristics (means \pm S.D.) included weight of 60 ± 12 kg and age of 22 ± 5 years. The study protocol was approved by the ethics committee of Nanjing University. Written informed consent was obtained from all participants prior to the study.

4.2. Fruits and juice

The fruits used in this study were purchased from local supermarkets. Watermelon juice was prepared fresh from watermelon pulp (seeds removed) using an electric juice extractor without any addition of water. Because the expression levels of plant miRNAs are significantly different in different types of fruits and because some plant miRNAs may be especially abundant in a specific fruit, the fruits (watermelon, apple, banana, orange, grape, mango and cantaloupe) were also assembled into a fruit mixture to balance the levels of plant miRNAs.

4.3. Reagents

The MirVana RNA isolation kits were purchased from Life Technologies. Synthetic miRNA standards were obtained from Invitrogen. The Total Exosome Isolation (from

plasma) kits were purchased from Invitrogen (Catalog Number: 4484450). The miRCURY LNA miRNA detection probes were obtained from Exiqon. The DIG luminescent detection kit was purchased from Roche Applied Science (Indianapolis, IN, USA).

4.4. Study design

The juice-drinking study and the fruit-eating study were performed on separate days. In the juice-drinking study, juice was extracted from watermelon pulp 1 h before ingestion, and each subject drank 2.5 L of juice. In the fruit eating study, each subject ate an equal portion of watermelon, apple, banana, orange, grape, mango and cantaloupe (2.5 kg in total). The subjects ingested the juice or fruit within 1 h at the study site the morning after an overnight fast. The subjects were not allowed to eat or drink again until the end point. The baseline blood samples were obtained 10 min before the administration of juice or fruit. The blood samples were collected into vacuum tubes containing EDTA at 0.5, 1, 3, 6 and 9 h after the juice and fruit were consumed.

4.5. RNA isolation

Venous blood samples (approximately 5 ml) were collected from each donor and placed in plasma separator tubes. The plasma was separated by centrifugation at $800 \times g$ for 10 min at room temperature, followed by a 15 min high-speed centrifugation at $10,000 \times g$ at room temperature to completely remove the cell debris. The supernatant plasma was recovered, and the RNA was isolated simultaneously to avoid the introduction of bias. The total RNA was extracted from 100 μ l of plasma using a phenol/chloroform purification protocol. Briefly, 100 μ l of plasma was mixed with 300 μ l of RNase-free water, then with 200 μ l of acid phenol. The mixture was vortexed vigorously and incubated at room temperature for 5 min. The mixture was then mixed with 200 μ l of chloroform and vortexed vigorously and incubated at room temperature for 10 min. After phase separation, the aqueous layer was mixed with 1.5 volumes of isopropyl alcohol and 0.1 volumes of 3 M sodium acetate (pH 5.3). This solution was stored at -20°C for 1 h. The RNA pellet was collected by centrifugation at $16,000 \times g$ for 20 min at 4°C . The resulting RNA pellet was washed once with 75% ethanol and dried for 10 min at room temperature. Finally, the pellet was dissolved in 20 μ l of RNase-free water and stored at -80°C until further analysis. The RNA concentration and quality (OD260/OD280 ratio) were measured using a Qubit fluorometer (Invitrogen) and a Quant-iT RNA Assay Kit (Invitrogen) according to the manufacturer's instructions. For comparison, the total RNA was also extracted from 100 μ l of plasma with Trizol LS and a MirVana RNA isolation kit according to the manufacturer's instructions.

4.6. qRT-PCR assay

The qRT-PCR procedure was carried out using TaqMan[®] MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, 5 μ l of total RNA was reverse-transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and stem-loop RT primers (Applied Biosystems). The reaction conditions were as follows: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Real-time PCR was performed using TaqMan miRNA probes (Applied Biosystems) on the Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions, including the no-template controls, were run in triplicate. After the reaction, the cycle threshold (C_T) values were determined using the fixed threshold settings, and the mean C_T values were determined from the triplicate PCR reactions. To calculate the expression levels of the target miRNAs, a series of synthetic miRNA oligonucleotides at known concentrations in water were also reverse-transcribed and amplified. The quantity of each miRNA was then calculated by referring to the standard curve. Based on the calculations of the concentrations of plant miRNAs in watermelon juice, the total uptake amount of each plant miRNA was determined. Based on the calculation of the area under the plasma concentration-time curve (AUC) of each plant miRNA, the total absorption amount was determined. The AUC was calculated using the trapezoid method. The absorption rate is the ratio of the total absorption amount to the total uptake amount.

4.7. Northern blot analysis

A sensitive non-radioactive northern blot method to detect miRNAs was performed [21]. The total RNA (including miRNAs) was collected from 100 μ l of watermelon juice or 4 ml of plasma using the phenol/chloroform purification protocol. The northern blot analysis was carried out using miRCURY LNA[™] microRNA Detection Probes with DIG-labeling (Exiqon) and a DIG luminescent detection kit (Roche) according to the manufacturer's instructions. Briefly, the total RNA isolated from 100 μ l of watermelon juice or 4 ml of plasma was dissolved in Gel Loading Buffer II (Ambion), heated at 95°C for 5 min, loaded onto a 15% TBE-Urea denaturing gel and transferred to a Zeta Probe plus membrane (Bio-Rad, Hercules, CA). After UV-crosslinking at an energy of 1200 mJ, the membranes were equilibrated with $2 \times \text{SSC}$ and prehybridized at 42°C for 1 h in ULTRAhyb-Oligo buffer (Ambion). Prior to hybridization, miRCURY[™] LNA detection probes were labeled using a DIG Oligonucleotide Tailing Kit 2nd Generation (Roche Applied Science, Indianapolis, IN). DIG-labeled LNA probes were hybridized to the membranes overnight at 37°C in ULTRAhyb-Oligo buffer (Ambion). Following

hybridization, the membranes were washed twice for 30 min in NorthernMax Low Stringency wash solution no. 1 (Ambion) at 42°C, rinsed for 5 min in the 1× Wash Buffer from the DIG wash and Block Buffer Set (Roche), blocked for 1 h in 1× Blocking Solution (Roche), incubated for 1 h in antibody solution (Anti-DIG-AP 1: 10,000 in 1× Blocking solution, Roche), washed twice for 15 min in 1× Wash Buffer and equilibrated by rinsing twice for 5 min each with 1× Detection Buffer (Roche). Then, following the instructions from the DIG Luminescent Detection Kit (Roche), the blots were incubated with the chemiluminescent substrate for alkaline phosphatase CSPD (Roche) and exposed to Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Piscataway, NJ).

4.8. MV isolation

Venous blood samples (approximately 5 ml) were collected from each donor and placed in plasma separator tubes. The plasma was separated by centrifugation at 800×g for 10 min at room temperature, followed by a 15 min centrifugation at 10,000×g at room temperature to completely remove the cell debris. The supernatant plasma was recovered, and the MV isolation was performed using the Total Exosome Isolation (from plasma) kit according to the manufacturer's instructions.

4.9. Data analysis

The data shown are presented as the mean±S.E.M. of at least three independent experiments. The statistical analyses were performed with SPSS 15.0 statistical software, and a *P* < .05 using a *t* test was considered significant.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jnutbio.2014.12.002>.

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