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An improvement of miRNA extraction efficiency in human plasma

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Abstract MicroRNAs (miRNAs) are short noncoding RNA molecules that control the expression of mRNAs associated with various biological processes. Therefore, deregulated miRNAs play important roles in the pathogenesis of diseases. Numerous studies are aimed at discovering biomarkers of diseases or determining miRNA functions by monitoring circulating miRNAs in various biological sources such as plasma and urine. However, the analysis of miRNA in such fluids presents problems related to accuracy and reproducibility because of their low levels in biological fluids. Therefore, better extraction kits and more sensitive detection systems have been developed for improved and reproducible analysis of circulating miRNAs. However, new extraction methods are also needed to improve the yield of miRNAs for their reliable analysis from biological fluids. The combination of yeast transfer RNA (tRNA) and glycogen as carrier molecules and incubation durations were optimized to maximize extraction efficiency. The extraction recovery using a combination of yeast tRNA and glycogen was approximately threefold more than that by using glycogen or yeast tRNA alone. In addition, reproducible and accurate analysis of miRNAs can be carried out after extraction using a combination of yeast tRNA and glycogen without an impact on plasma components.

Keywords miRNA · Glycogen · Yeast tRNA · Extraction efficiency

Introduction

MicroRNAs (miRNAs) are short non-coding single-stranded RNA molecules, approximately 22 nucleotides in length, which regulate the expression and/or degradation of mRNA [1, 2]. miRNAs have been implicated in different physiological and pathological processes, including metabolism, proliferation, apoptosis, differentiation, development, viral infection, and cancer [1, 2]. Specifically, miRNAs in plasma have been evaluated as diagnostic and therapeutic biomarkers for various diseases [3, 4].

The levels of circulating miRNAs in biological fluids are measured after extraction by commercial RNA isolation kits, followed by quantitative real-time polymerase chain reaction (qRT-PCR), northern blotting, or microarray analysis. More recently, some research groups have also examined the use of capillary electrophoresis with laser-induced fluorescence (CE-LIF) for quantifying miRNAs in plasma [5–8]. However, the comparison of miRNA levels in the plasma or serum of normal and diseased persons is difficult, owing to insufficient assay accuracy and precision involving the trace amounts present in plasma or serum. Therefore, the method for extraction of miRNA needs to be improved to maximize recovery, especially that of low-abundance miRNAs in plasma. The optimization and evaluation of different methods of extracting circulating miRNA using various kits is an ongoing process.

Typically, miRNA extraction involves three basic steps: lysis, organic extraction, and precipitation. Among these

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steps, precipitation is the most critical step with respect to improvement of extraction efficiency [9]. Therefore, appropriate precipitation conditions are essential to maximize the recovery of miRNA from plasma. However, precipitation of total RNA, including miRNAs, is generally difficult using only commercial RNA isolation kits, owing to the low total miRNA content in plasma. Therefore, the use of carriers such as glycogen, linear acrylamide, and yeast transfer RNA (tRNA) is common for improving the efficiency and reproducibility of plasma miRNA extraction [9–11]. These carriers are insoluble in isopropanol and form a precipitate that traps RNA, including miRNA. During centrifugation, a visible pellet is formed, which facilitates miRNA extraction. This study clarified the effects of the type and amount of carriers, such as glycogen and tRNA, on the efficiency of miRNA extraction from plasma. We chose miRNA155 as a target miRNA because its levels in plasma are significantly different between normal subjects and subjects with various diseases, including cardiovascular and inflammatory diseases, and it is an important biomarker candidate [12–14]. miRNA155 was extracted from plasma, and the extraction efficiency by a carrier was assessed using both CE-LIF and qRT-PCR.

Experiments

Chemicals and materials

Normal and lipidemic human pooled plasmas collected in K3-EDTA were purchased from Biochemed Services (Winchester, VA, USA). Normal human plasma was obtained from normal healthy adult donors aged between 20 and 30 years. Lipidemic plasma was obtained from normal healthy adult donors with a total cholesterol level of 300 mg/dl. All donors also denied a history of renal, endocrine, hepatic, autoimmune, and neoplastic diseases. Although the donors stated that they were healthy at the time of collection, the plasma was noted to be moderately lipidemic. Rat plasma samples collected in K3-EDTA from three individual female Sprague Dawley rats were obtained from CHA University (Seongnam, South Korea). Adult female Sprague Dawley rats (200–220 g) were purchased from Orient Bio, Inc. (Seongnam, South Korea). The animals were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of CHA University (IACUC150058), Seongnam, South Korea. Rat plasma samples were thawed and pooled prior to RNA extraction. A fluorescence-labeled single-stranded DNA (ssDNA) oligonucleotide probe with 5′-carboxyfluorescein phosphoramidite (6-FAM) [miR-155 (5′-ACCCCTATCACGATTAGCATTAA-6-FAM-3′) and miR-27a (5′-GCGGAACTTAGCCACTGTCAA-6-FAM-3′)] was purchased from Cosmogenetech (Seoul, South Korea). Synthetic HPLC-grade miRNAs [miR-155 (5′-

UUAAUGCUAAUCGUGAUAGGGGU-3′) and miR-27a (5′-UUCACAGUGGCUAAGUUCCGC-3′)] were purchased from ST Pharm (Seoul, South Korea). TRIzol™ LS reagent and DNase/RNase-free water were obtained from Invitrogen (Carlsbad, CA, USA). Muscle glycogen and yeast tRNA were purchased from Invitrogen (Carlsbad, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

RNA extraction from plasma

Synthetic miRNA (1 nM miR-155) was spiked into 250 μL of plasma and homogenized with 750 μL of TRIzol™ LS reagent (Invitrogen, Carlsbad, CA, USA), followed by 200 μL of chloroform. Each sample was vortexed for 30 s and incubated at room temperature for 5 min. Phase separation was performed by centrifuging the samples at $12,000 \times g$ for 15 min at 4 °C. Four hundred microliters of the upper aqueous phase was transferred to a new tube. Glycogen (5 mg/mL; Invitrogen, San Diego, CA, USA) or tRNA (10 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) for RNA precipitation was added to the aqueous phase before being mixed with 100 μL of 3 M sodium acetate and 500 μL of isopropanol. The tube was vortexed for 30 s and incubated in − 80 °C for 1 h to allow the RNA precipitation. The tube was then centrifuged at $20,000 \times g$ for 30 min at 4 °C. After centrifugation, the supernatant was discarded, and the RNA pellet was dissolved with 30 μL of RNase-free water.

Total RNA concentration and quality

Total RNA concentration and quality were assessed using NanoDrop Lite Spectrophotometer 120V (Thermo Fischer Scientific, MA, USA) at the absorbance of 260 and 280 nm. The total RNA concentration was estimated by measuring the absorbance at 260 nm, and the A_{260}/A_{280} ratios were used to assess the presence of contaminants.

qRT-PCR

The extracted miRNA from plasma was analyzed using qRT-PCR. Isolated RNA including miRNA was used for complementary DNA (cDNA) synthesis. The transcription was performed in a reaction mixture, containing a 500 ng RNA sample using Mir-X™ miRNA First-Strand Synthesis and SYBR qRT-PCR Kit from Clontech (Mountain View, CA, USA) according to the manufacturer's protocol. cDNA synthesis was conducted for 1 h at 37 °C and then terminated by heating at 85 °C for 5 min. cDNA was diluted ten times and mixed with SYBR qPCR Mix containing 0.5 μL of 10 μM miR-155-specific primers (5′-TTAATGCTAATCGTGATAGGGGT-3′) and universal mRQ 3′ reverse primer from the Mir-X™ miRNA qRT-PCR SYBR Kit. PCR was performed for 10 s at 95 °C, 5 s at 95 °C, and 20 s at 60 °C for 40 cycles and

finalized by melting a curve for 5 s for each 0.5 °C. The CFX Connect™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) was performed for both cDNA and real-time PCR reactions. Finally, the samples were analyzed using CFX Manger™ software (Bio-Rad).

Capillary electrophoresis

miRNA was analyzed by CE-LIF-based hybridization with a fluorescence-labeled DNA probe. For hybridization, 6-FAM-labeled DNA probes (1 nM) and synthetic miRNAs (1 nM) were mixed with the hybridization buffer (50 mM Tris–acetate, pH 8.0, containing 50 mM NaCl, 0.1 mM EDTA, and 1% Triton X-100). The labeled DNA probes were dissolved in elution buffer (10 mM Tris–Cl buffer, pH 8.5) from a QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK). The samples were incubated in a thermal cycler (Eppendorf) for denaturation at 95 °C for 5 min, followed by a renaturation step at 40 °C for 15 min prior to introduction into the capillary.

CE condition was based on our previously published paper [8]. Briefly, the extracted miRNA from plasma was analyzed using a PA800 plus CE system (Beckman Coulter, Fullerton, CA, USA) with a LIF detector. Fluorescence was detected by excitation at 488 nm using a 3-mW argon-ion laser and emission through a 520-nm emission filter at a rate of 4 Hz. Separations were performed using an untreated capillary (Beckman Coulter), which had a 75 μm inner diameter and 50 cm length (40 cm effective length). Separations were performed using a running buffer of 125 mM Tris–borate (pH 10.0) containing 2.5 M urea at 25 °C by applying 14 kV while the sample compartment was maintained at 25 °C. Samples were introduced hydrodynamically at 0.5 psi for 15 s. Electropherograms were analyzed using 32 Karat software (Beckman Coulter, Fullerton, CA, USA). The relative fluorescence intensity of miRNA represents the ratio of the peak heights of miRNA and DNA probe complex over the internal standard peak height. All peak heights of miRNA were normalized by dividing them by the height of the internal standard, fluorescein. The vertical axis represents the ratio of the complex peak height over internal standard peak height. Fluorescein (1 nM) as an internal standard (IS) was added to all samples.

Statistical analysis

All the means were expressed with their standard deviation (mean ± SD). An unpaired Student's t test was performed for comparison between two groups, and the data was also analyzed by one-way analysis of variance (ANOVA) among multiple groups. The differences were considered to be significant at $P < 0.05$. All the statistical calculations were performed with GraphPad Prism software (version 5.01; Graph Pad Software, California, USA).

Results and discussion

Effect of carriers on the extraction of plasma miRNA

The analysis of circulating miRNA in biological fluids is gaining importance in clinical and pharmaceutical settings because miRNAs can be effective diagnostic or therapeutic biomarkers. Consequently, there is an increase in the analysis of circulating miRNAs from biological samples for functional studies of miRNAs and for potential use in drug discovery. However, comparison of miRNA levels is difficult because these vary depending on sample composition and experimental conditions of the extraction method and analysis. This variability in extracted miRNA levels could be attributed to trace amounts of endogenous miRNA in biological samples. Consequently, each process is being fine-tuned to obtain reliable results. Specifically, there is an increase in studies examining the extraction of miRNAs from biological fluids (including plasma) and, in most cases, the evaluation of the recovery of miRNA focuses on the extraction method using several
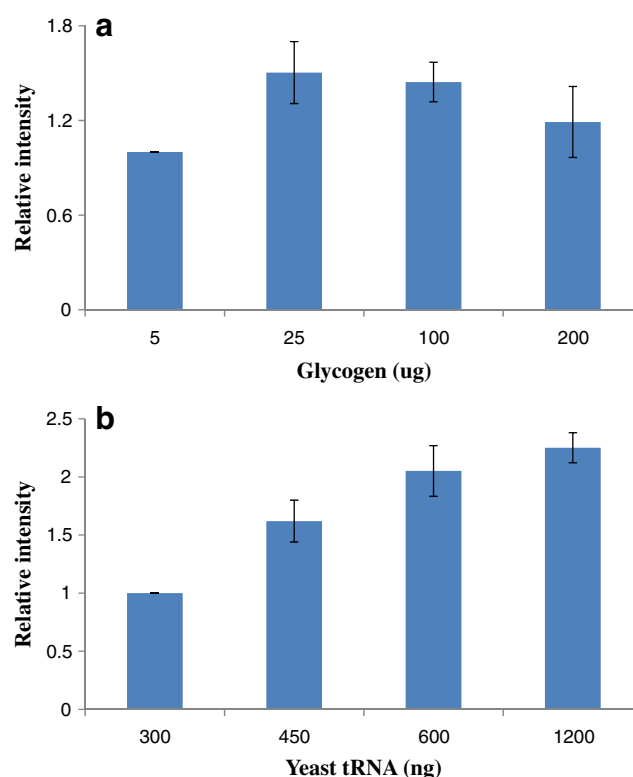
Fig. 1 Effects of amount of (**A**) glycogen (5~200 μg) and (**B**) tRNA (300~1200 ng) as a carrier on extraction efficiency of miRNA. The data was obtained using CE-LIF after extraction of spiked-in exogenous miRNA155 (1 nM) from plasma. Error bars indicate the standard deviations from three independent experiments in the same condition. The vertical axis represents the relative fluorescence intensity of fluorescence-labeled ssDNA probe, the extracted miRNA155 complex peak over the fluorescence-labeled ssDNA probe, and the extracted miRNA155 complex peak after extraction using 5 μg glycogen or 300 ng tRNA as a carrier
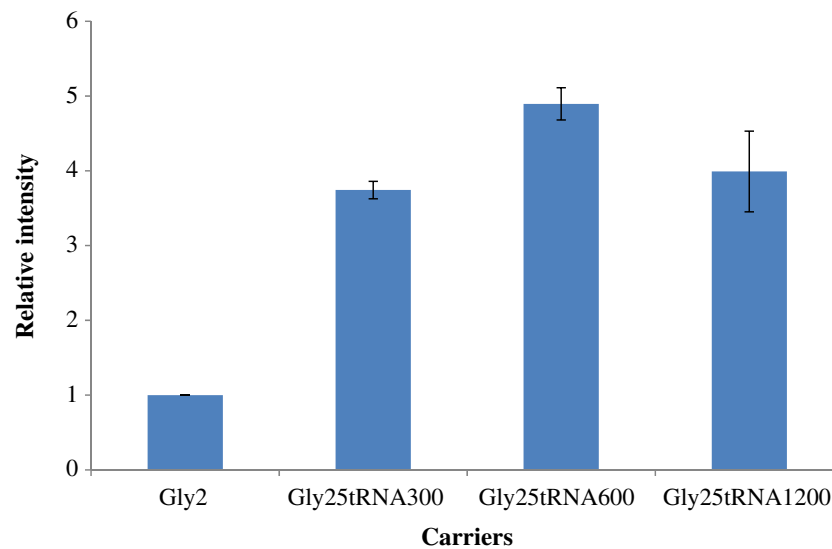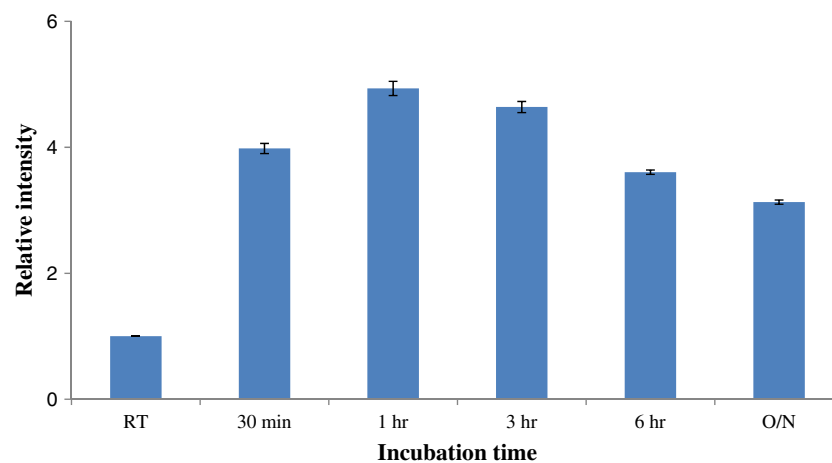
Fig. 2 Effects of the ratio of tRNA to glycogen on extraction efficiency of miRNA. The data was obtained using CE-LIF after extraction of spiked-in exogenous miRNA from plasma. Error bars indicate the standard deviations from three independent experiments in the same condition. The vertical axis represents the relative fluorescence intensity of fluorescence-labeled ssDNA probe, the extracted miRNA155 complex peak over fluorescence-labeled ssDNA probe, and the extracted miRNA155 complex peak after extraction using 25 μg glycogen

extraction kits. This study aimed to establish and optimize conditions by simple modification of extraction processes or reagents for the efficient extraction of miRNAs from plasma. In this study, the TRIzol™ LS-treated plasma sample was extracted with chloroform and the supernatant precipitated with isopropanol. The miRNA was recovered in the form of a concentrated pellet. The minute amounts of total miRNAs in plasma cause the formation of a very small pellet and consequently reduce the yield and reproducibility of miRNA extractions. To improve RNA pellet formation during precipitation, we tested the amount and type of carriers and incubation conditions. The addition of carriers is known to promote RNA

pellet formation and is therefore a common step in the extraction of miRNA from plasma samples. Although glycogen, tRNA, and linear acrylamide are commonly used as carriers, only a few studies have reported the effect of carrier type or concentration on extraction efficiency of miRNAs from plasma [15, 16]. Therefore, in this study, the effect of carriers on the extraction of miRNA from plasma was evaluated and the type of carrier used and its amount were optimized for maximizing the extraction efficiency. Only glycogen and tRNA were tested because the pellet formed using linear acrylamide may not adhere tightly to plasticware and be readily lost [17]. Currently, the amount of glycogen or tRNA used varies with

Fig. 3 Effects of incubation time and temperature on extraction efficiency of miRNA. The data was obtained using CE-LIF after extraction of spiked-in exogenous miRNA from plasma. Error bars indicate the standard deviations from three independent experiments in the same condition. The incubation at RT was conducted for 10 min, and the incubation at −80 °C was conducted for 0.5, 1, 3, and 6 h and overnight. The vertical axis represents the relative fluorescence intensity of fluorescence-labeled ssDNA probe, the extracted miRNA155 complex peak over fluorescence-labeled ssDNA probe, and the extracted miRNA155 complex peak after incubation at RT for 20 min

the method employed for extracting RNA from plasma [10, 15, 18, 19]. Therefore, in this study, the optimal amount of carrier was determined by analyzing the effect of varying amounts of glycogen or tRNA on the extraction efficiency. It was observed that the recovery of miRNA gradually increased with increasing amounts of both glycogen and tRNA. A further increase in the recovery of extracted miRNA was not significant at amounts of glycogen higher than 25 μg, and the recovery decreased at amounts higher than 75 μg. Moreover, the miRNA pellet was not easily solubilized at glycogen amounts higher than 100 μg (Fig. 1A). The recovery pattern was similar, whether CE-LIF or qRT-PCR was used for detection. Therefore, we concluded that 25 μg was the optimal amount of glycogen for high extraction efficiency of miRNA from plasma. Similarly, when tRNA was used as the carrier, the recovery of miRNA increased with increasing amounts of added tRNA but the increase was not significant for more than 600 ng, as shown in Fig. 1B. In addition, the pellet was not formed until tRNA was used at amounts higher than 3000 ng. These results showed that increasing the amount of glycogen or tRNA alone resulted in a limited improvement in the extraction efficiency of miRNA from plasma. Therefore, to further increase the efficiency of miRNA extraction, we used a mixture of glycogen and tRNA as a carrier. In this study, the maximum miRNA extraction efficiency was obtained by varying the amount of tRNA from 300 to 1200 ng while maintaining the amount of glycogen at 25 μg. As shown in Fig. 2, the intensity of extracted miRNA gradually increased with increasing amounts of tRNA but decreased at amounts higher than 600 ng. In addition, it was observed that the combination of tRNA and glycogen was more effective than glycogen or tRNA alone. Therefore, we concluded that to maximize the extraction efficiency, the optimal composition and amount of carrier was a combination of glycogen and tRNA at 25 μg and 600 ng, respectively.

Assessment of the quality of RNA extracted from plasma

In order to assess the quality of RNA extracted from plasma using the combination of tRNA and glycogen, the RNA samples were analyzed using a NanoDrop spectrophotometer. The A_{260}/A_{280} ratio was used to assess the purity of the RNA sample. The A_{260}/A_{280} values for RNA extracted using the combination of tRNA and glycogen were 1.95 ± 0.04, and those for tRNA or glycogen alone were 1.74 ± 0.00 and 1.66 ± 0.01, respectively. These data indicated that the purity of the RNA samples extracted using the combination of glycogen and tRNA was increased, related to the use of glycogen or tRNA alone as a carrier. In addition, the yield of the RNA in plasma obtained using the combination of glycogen and tRNA was approximately 2.5-fold and 2.3-fold higher than that obtained using glycogen or tRNA alone, respectively.

These results showed that the combination of tRNA and glycogen greatly improved the yield and purity of RNA and effectively maximized the extraction of miRNA from plasma when using the TRIzol™ LS. Therefore, the above combination was used to determine optimal incubation conditions for miRNA extraction.

Optimization of incubation conditions for the efficient extraction of miRNA

Along with the presence of carriers, the incubation process also influences the formation of the pellet and therefore efficient extraction of RNA from plasma. Generally, to improve the extraction recovery of miRNA from plasma, a solution containing RNA is incubated for more than 2 h on ice to promote flocculation of the nucleic acids so they form a pellet upon centrifugation [20]. However, in principle, at low temperatures, the precipitation efficiency can decrease because of the increased dielectric constant of the alcohol solution and
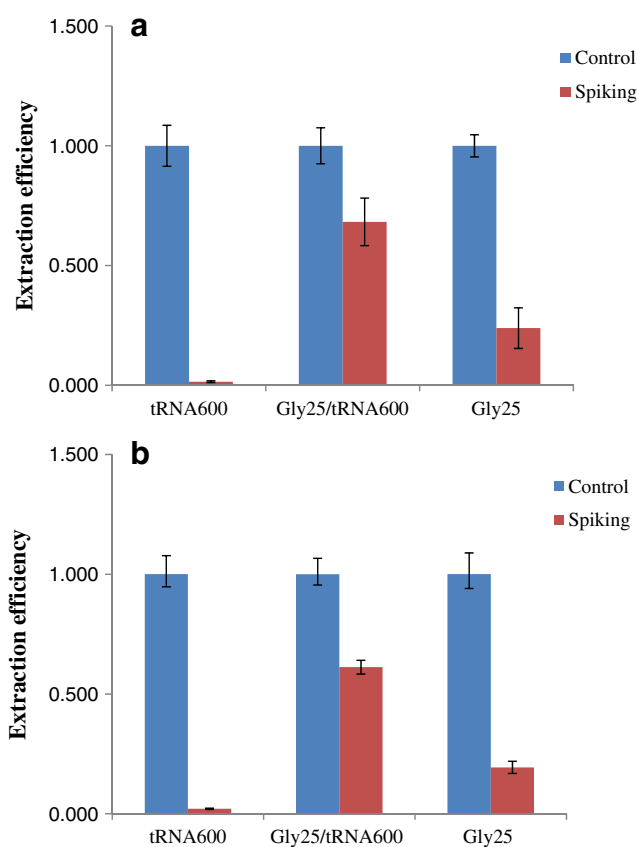
Fig. 4 Recovery of spiked-in exogenous miRNA155 extracted from the plasma sample using the combination of tRNA and glycogen and glycogen or tRNA alone. The recovery was obtained using (**A**) CE-LIF and (**B**) qRT-PCR after extraction of spiked-in exogenous miRNA from plasma. The vertical axis represents the relative fluorescence intensity or Cq value of spiked-in exogenous miRNA155 over control. Control is the level of spiked-in exogenous miRNA155 recovered after being added to the pellet after the extraction step
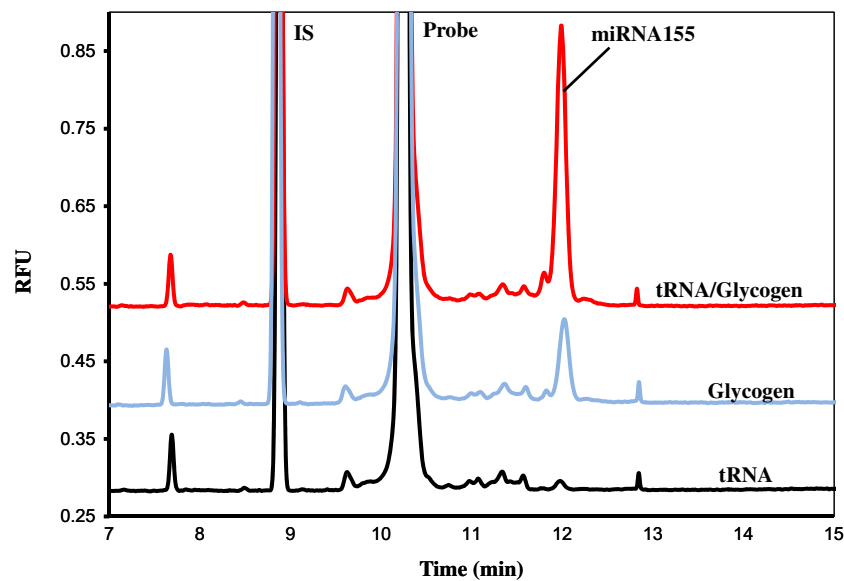
Fig. 5 Electropherograms of miRNA155 extracted from the plasma sample using the combination of tRNA and glycogen and glycogen or tRNA alone. Each sample was incubated with a 1-nM 6-FAM-labeled ssDNA probe in hybridization buffer. Capillary electrophoresis conditions: capillary = 75 μm id × 30/40 cm, separation voltage = 14 kV, injection = 15 s at 0.5 psi, and buffer = 125 mM Tris–borate buffer (pH 10) containing 2.5 M urea. The hybridization was conducted at 40 °C for 15 min after pre-incubation at 95 °C for 5 min in 50 mM Tris–acetate buffer, pH 8.0, containing 50 mM NaCl, 0.1 mM EDTA, and 1% Triton X-100. The fluorescein (1 nM) was used as the internal standard (IS)

co-precipitation of salts may occur. Therefore, the incubation time needs to be adjusted for effective extraction and rapid analysis. In order to optimize incubation time, we tested the effects of various incubation times on the recovery of miRNA at RT and − 80 °C. Plasma samples containing the combination of tRNA and glycogen as a carrier were incubated for 10 min at room temperature or for longer than 30 min at − 80 °C. miRNA was recovered as pellets and detected by CE-LIF. As shown in Fig. 3, low temperatures (− 80 °C) and prolonged incubation times resulted in greater yields of miRNA compared to those obtained at room temperature. Incubation at − 80 °C for 1 h appeared to be optimal, while prolonged incubation (overnight) resulted in a marginal reduction in yield. Relative to the results obtained with the commonly used protocol, miRNA recovery under optimized conditions was increased 1.5-fold and incubation time decreased to 1 h (from overnight).

Effect of plasma volume on extraction efficiency of miRNA

We also tested the effect of different volumes of plasma on efficiency of miRNA extraction using TRIzol LS with glycogen and the combination of tRNA and glycogen as carriers. We determined the yield of miRNA from sample solutions containing different volumes of plasma using both CE-LIF and qRT-PCR. When 25 μg glycogen was used as a carrier, the recovered miRNA levels increased with increasing plasma volumes, indicating that the components in plasma affect the extraction efficiency of miRNA (see Electronic

Supplementary Material (ESM) Fig. S1). This result may be due to various components in plasma such as salts affecting the precipitation of RNA. These differences in the extraction efficiency based on sample composition can lead to inconsistent results for endogenous miRNA levels. However, when a combination of tRNA and glycogen was used, the exogenous miRNA levels did not show significant changes with changes in plasma volume, by both miRNA detection methods, CE-LIF and qRT-PCR. This result shows that the proposed optimization step would be useful for accurate screening of miRNAs in biological samples because it would help reduce result variations based on sample composition. The effect of presence of lipids on extraction efficiency was tested using normal and lipidemic plasma containing 250 mg/mL cholesterol. Similar miRNA extraction efficiency was observed for both plasma samples (ESM Fig. S2). Thus, we concluded this optimized extraction technique allows efficient miRNA recovery, irrespective of the volume and composition of the biological fluid.

Evaluation of the extraction efficiency of miRNA155 from plasma using optimal extraction conditions

To evaluate the efficiency of miRNA extraction using the optimal conditions determined in this study, we compared the recovery of spiked-in exogenous miRNA155 from plasma using tRNA or glycogen alone and a combination of tRNA and glycogen. The pellets of total RNA obtained after extraction under the three conditions were dissolved,

and miRNA was detected using qRT-PCR and CE-LIF (Fig. 4A, B). Recovery of miRNA155 in each case was calculated with respect to the levels of an equivalent amount of miRNA155 that was added to the pellet after the extraction step in each case. miRNA recovery using the combination of tRNA and glycogen was threefold higher (68.2 vs 23.9 vs 1.5%) than that obtained using glycogen or tRNA alone. And, the t test results showed no significant difference between two detection methods used at the 5% significance level (0.109, 0.315, and 0.429). Additionally, to confirm the repeatability of high extraction efficiency using a combination of tRNA and glycogen with different plasma samples, we compared the extraction efficiency of miRNA among the samples extracted from two different lots of human plasma and one rat plasma using tRNA or glycogen and a combination of tRNA and glycogen as a carrier. The result showed that the combination of tRNA and glycogen as a carrier led to extracting a higher quantity of miRNA in all the samples tested as expected (ESM Fig. S3). Moreover, the detection limit of miRNA155 extracted from plasma using the combination of tRNA and glycogen was 44 pg/mL and improved approximately 2.5-fold compared to that obtained using glycogen alone in the CE-LIF system. This combination of tRNA and glycogen showed high extraction efficiency of miRNA27a which has different sequences and properties with miRNA155 (ESM Fig. S4). Also, the recovery of miRNA155 from plasma using a combination of tRNA and glycogen was similar a"t three spiked-in miRNA155 concentrations (Table S1). These results showed that the developed extraction condition of miRNA would be capable of application to effective extraction of miRNA from plasma regardless of sequence and concentration of miRNA. Taken together, these data confirm that the combination of tRNA and glycogen significantly improved the extraction efficiency of miRNAs from plasma without any interference or degradation.

Conclusion

Our study focused on the development of a carrier-assisted precipitation method in order to increase the recovery of total RNA, including miRNA. In the present study, we developed a method to improve miRNA extraction efficiency through the combined use of optimal amounts of glycogen and tRNA. Furthermore, the efficiency of extraction was analyzed and evaluated using CE-LIF, which is used to quantitate miRNA, involving specific hybridization between target miRNA and fluorescently labeled single-stranded DNA probe without interference from co-precipitated components in the extract.

As shown in Fig. 5, the miRNA peak was the highest when the combination of tRNA and glycogen was used as a carrier. This result was found to be reliable, as the qRT-PCR data were similar to those obtained using CE-LIF.

In conclusion, our findings demonstrate that the extraction of endogenous miRNA in biological samples can be significantly improved by a combination of the carriers tRNA and glycogen. This result can be useful and effective for performing clinical or therapeutic and biological studies in the determination of endogenous miRNAs isolated from plasma.

Compliance with ethical standards

Human plasma Human pooled plasmas collected in K3-EDTA were purchased from Biochemed Service (Winchester, VA, USA).

Rat plasma Rat plasma samples collected in K3-EDTA from three individual female Sprague Dawley rats were obtained from CHA University (Seongnam, South Korea). The animals were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of CHA University (IACUC150058), Seongnam, South Korea.

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