



A Simplified Method for RNA Isolation from Biofabricating Hydroxyapatite Scaffolds and Identification of Appropriate Reference Genes

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Abstract

Purpose To validate a simplified RNA isolation method from biofabricating hydroxyapatite (HAp) scaffolds seeded with mesenchymal stem cells (MSCs) and to identify the appropriate reference gene.

Methods Ten MSCs-HAp composites were used for RNA isolation by methods based on simplified homogenization steps and column-based purification procedures, while the remaining RNA (n = 13) was extracted by traditional single-step isolation methods. The differences between the two procedures regarding the operation time, RNA quantity and quality were evaluated. Quantitative real-time PCR (qRT-PCR) analysis was performed to identify the appropriate reference gene.

Results The simplified method showed significant superiority in operation time ($P < 0.001$), RNA concentration ($P < 0.001$), A260/280 ratio ($P = 0.005$) and A260/230 ratio ($P < 0.001$). The average integrity number and 28 s/18 s ratio of RNA yielded by the simplified method were 9.1 ± 0.2 and 1.3 ± 0.1 , respectively. The qRT-PCR analysis results indicated that the cycle threshold (Ct) values of GAPDH were significantly higher than those of the remaining 2 reference genes (ACTB and RPL13A) in the RNA samples obtained by the simplified and traditional methods ($P < 0.05$). The standard deviations of the Δ Ct value (the difference between the Ct value and the minimum) of ACTB were higher than those of GAPDH or RPL13A, regardless of the RNA isolation method.

Conclusion The simplified method could extract intact RNA from biofabricating MSCs-HAp scaffolds and was superior to the traditional single-step procedure in operation time, RNA quantity and quality. GAPDH was identified as the most appropriate reference gene in MSCs-HAp scaffold composites due to its high quantity and good stability.

Keywords Hydroxyapatite scaffold · RNA · Isolation · Quantity · Purity · Integrity

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1 Introduction

Hydroxyapatite (HAp) scaffolds are considered to be an efficient artificial bone substitute material due to their similar structure and good osteoconduction and the feasibility of mesenchymal stem cell (MSCs) adhesion [1–3]. Previous studies have attempted to improve the osteoinductive ability of HAp scaffolds with the addition of bioactive factors or by gene modification [4–6]. Understanding the mechanisms of gene regulation in the biofabrication of MSCs-HAp scaffold composites could help improve their biological properties. Quantitative real-time PCR (qRT-PCR) results indicate the level of gene expression; therefore, isolation of a large quantity of highly pure and intact RNA from the MSCs-HAp scaffold composites is needed.

Previous studies have indicated that the HAp scaffold can be homogenized into powders using a hammer, mortar and pestle or ultrasound concussion to extract RNA within acid guanidinium thiocyanate-phenol-chloroform [7–9]. However, these techniques are limited by potential RNA degradation and sample loss [7–9]. These complications may be attributed to the multiple time-consuming homogenization procedures. Furthermore, the presence of HAp powders introduces the potential of RNA contamination, and the sample loss could be attributed to the fact that HAp may absorb the RNA [10, 11]. Considering that the structure of the MSCs-HAp scaffold is fragile and porous [12, 13] and embedded with less extracellular matrix, whether the time-consuming homogenization procedures could be replaced by simplified steps, such as grounding the scaffold into large fragments instead of powders and vortexing the large fragments in an acidic RNA extraction solution to isolate RNA, remains to be elucidated.

In addition, high-purity RNA is necessary for further analysis. The RNA purification procedures in the traditional single-step isolation methods were finished by the removal of supernatant detergent following centrifugation. However, the RNA could not be fully purified due to the mixture with the detergent, and further time-consuming purification procedures may lead to RNA degradation. Several reports indicated that the RNA filtration column-based purification method is more time-efficient and could be used in tissues from different sources; however, whether the column could be used to purify RNA isolated from HAp scaffolds remains uncertain [14, 15].

In addition to RNA quantity and quality, the selection of an appropriate reference gene is necessary for accurate qRT-PCR results. To our knowledge, no studies have clearly discussed the selection of appropriate reference genes in MSCs-HAp composites. In this study, we aimed to compare our simplified method based on convenient homogenization procedures and column-based purification

steps with traditional single-step RNA isolation procedures to extract RNA from MSCs-HAp scaffold composites in regards to operation time and RNA quantity, quality and integrity and to identify the appropriate reference gene.

2 Materials and Methods

This study was approved by the Institutional Review Board of the Seventh Affiliated Hospital of Sun Yat-sen University (Application no: 2019110401).

2.1 Preparation of MSCs-HAp Scaffold Composites

Cleaned bovine vertebrae were drilled into cylindrical shapes 6.5 mm (mm) in diameter and 10 mm in height by a hollow drill and then calcined to HAp scaffolds according to a previous protocol [16]. Briefly, the cylindrical vertebra was heated at 900 °C for 6 h and then sintered at 1300 °C for 1 h. After cooling to room temperature (RT), HAp scaffolds approximately 5 mm in diameter and 9 mm in height were obtained. The calcined HAp scaffolds were sterilized using ethylene oxide.

The Wharton's jelly of umbilical cords from three human samples was used to isolate MSCs using the adhesion culturing method [17] and cultured in serum-free Prim® human MSCs medium (Premedical Laboratories, China) containing 1% primocin (Invitrogen, USA) at 37 °C in a 5% CO₂ humidified incubator. MSCs were passaged when cell confluence reached approximately 80%, and MSCs of passage 4 were used for cell seeding in the HAp scaffolds.

MSCs suspensions with a density of 1×10^7 cells/ml (ml) were seeded on the HAp scaffolds according to previous methods [5]. Briefly, the scaffolds were soaked completely within cell suspension and then treated with a low atmosphere pressure condition for 2 min (min) to distribute cells into the inside of the scaffolds. After cell seeding for 4 h, the MSCs-HAp scaffold composites were transferred to 6-well plates with DMEM/F12 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA). The MSCs-HAp scaffold composites were used for RNA isolation after 14 days of culture (Fig. 1).

2.2 RNA Isolation by the Simplified Method

Figure 2 shows the simplified RNA isolation procedures. The MSCs-HAp scaffold composite was cut into large fragments approximately 2.5 mm in length, 2.5 mm in width and 2.5 mm in height using RNase-free microsurgical scissors. The large fragments were transferred to a new 2 ml RNase-free tube containing 1 ml chilled TRIzol Reagent (Invitrogen, USA), while the remaining small fragments and powders were discarded. The fragments were incubated in

Fig. 1 Gross view of MSCs-HAp scaffold composites. **A** MSCs under the microscope; **B** porous structure of calcined HAp scaffold. **C** View of MSCs-HAp scaffold composites under a microscope

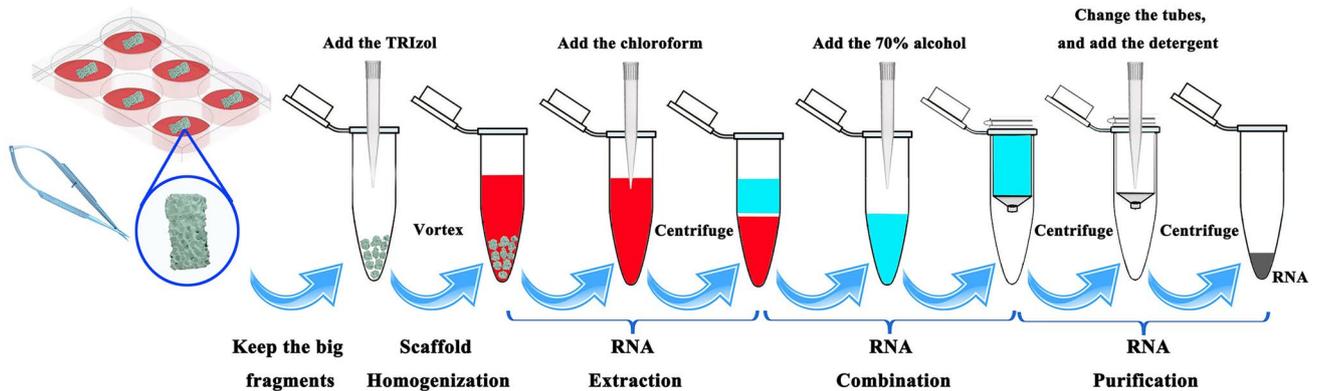


Fig. 2 Simplified RNA isolation procedures

TRIzol for 10 min at RT, followed by vortexing for 10 seconds (s). Then, TRIzol was transferred to a new RNase-free tube.

Chloroform (Sigma, USA) was added at a proportion of 100 microlitre μl (μl)/1 ml TRIzol; then, the mixed liquid was vortexed for 10 s, incubated for 1 min at RT and centrifuged at $12,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 15 min. The resulting aqueous phase was transferred to a new RNase-free tube containing 70% ethanol (Aladdin, China) with the same volume of aqueous phase and mixed well by pipetting.

Then, a spin column (Beyotime, China) was used to purify RNA following the manufacturer's instructions. Briefly, the mixed liquid was transferred to the column and then centrifuged at $12,000\times g$ for 30 s to absorb RNA on the column. Then, the detergent was added to the column, and it was centrifuged at $12,000\times g$ for 30 s to purify the RNA. Finally, the eluent was added to the column, and RNA was collected from the column following centrifugation at $16,000\times g$ for 2 min and recovered with 30 μl RNase-free H_2O (Biosharp, China).

2.3 RNA Isolation by the Traditional Single-Step Method

RNA was isolated according to a previous protocol (Fig. 3) [7]. Briefly, the composite was transferred to an RNase-free

tube containing 1 ml TRIzol reagent, crushed into small fragments by an RNase-free hammer, and then ground to powder by ultrasound concussion (40 Hertz, 1 min, $4\text{ }^{\circ}\text{C}$). After incubation at RT for 10 min, TRIzol was transferred to a new RNase-free tube containing chloroform in a proportion of 100 μl /1 ml TRIzol. The resulting aqueous phase was obtained from the mixed liquid after vortexing for 10 s, incubating for 1 min at RT and centrifuging at $12,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 15 min. The aqueous phase was transferred to a new RNase tube and added to the same volume of isopropanol (Sigma, USA). After incubating at RT for 10 min, the mixed liquid was centrifuged at $12,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 10 min, and finally, RNA was obtained from the bottom of the tube.

Following removal of the supernatant, 75% ethanol was added to the tube, and it was vortexed for 10 s at RT and centrifuged at $7500\times g$ at $4\text{ }^{\circ}\text{C}$ for 5 min to purify the RNA. Then, the RNA was warmed at RT for 15–20 min following the removal of supernatant and was finally recovered with 30 μl RNase-free H_2O .

2.4 Evaluation of RNA Concentration, Purity and Integrity

The concentration and purity of RNA were evaluated using a NanoDrop 2000 instrument (ThermoFisher, USA). The

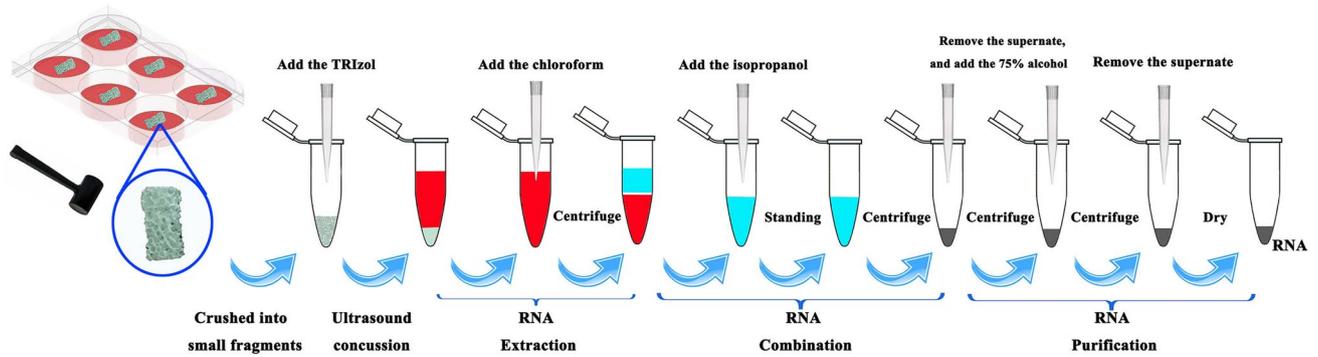


Fig. 3 Traditional RNA extraction methods

Table 1 The sequences of primers used in the experiments

Species	Gene	Primers	Sequences (5' to 3')
Human	ACTB	Forward	CAGGGCGTGATGGTGGGCA
		Reverse	CAAACATCATCTGGGTCATCTTCTC
Human	GAPDH	Forward	TTGGTATCGTGGAAGGACTCAT
		Reverse	TGTTCTGGAGAGCCCCGC
Human	RPL13A	Forward	CCAATAGGAAGAGCAACCA
		Reverse	GAGTATATGACCAGGTGGAA

A260/280 and A260/230 ratios of approximately 2 indicated that the RNA was free from contaminants.

The RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) following the manufacturer's protocol. Briefly, RNA fragments could be separated electrophoretically based on size into a set of microchannels contained in the chip. The RNA integrity number (RIN) value, which ranged from 10 (intact RNA) to 1 (degraded RNA), and the calculated ribosomal ratio (28 s/18 s), which indicated that RNA was not degraded if the value was approximately 2, were obtained using the software [18].

2.5 Identification of Appropriate Reference Gene

Reverse transcription of RNA was performed using SuperScript IV VILO Master Mix (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. For each human MSCs sample ($n=3$), the cDNA obtained from RNA isolated by the same method (simplified or traditional) was mixed and used for qRT-PCR analysis. Three commonly used genes (ACTB, GAPDH and RPL13A) were evaluated to identify the appropriate reference gene. The primers (Qingke, China) are shown in Table 1.

qRT-PCR analysis was performed using a CFX96 Touch™ RealTime PCR Detection System (Bio-Rad

Laboratories, USA) and PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA). The qRT-PCR tests of each gene were repeated three times, and the mean cycle threshold (Ct) values were used for the statistical analysis. A high Ct value indicates a low gene quantity in the RNA samples.

The stability of reference genes was evaluated by the Δ Ct value, which represented the difference between the Ct value and the minimum. As previously described [19], a high standard deviation (SD) of Δ Ct values is a sign of low stability of the gene.

2.6 Statistical Analysis

Statistical analysis was performed using the statistical package SPSS 13.0 (SPSS, USA).

Data are expressed as numerical variables, frequencies, and percentages, with means and standard deviations. One-way ANOVA was used to compare the difference among three human MSCs samples regarding the number of HAP scaffolds used for RNA isolation by two different methods and to assess the difference in Ct and Δ Ct values among ACTB, GAPDH and RPL13A genes in RNA samples obtained by the simplified methods or traditional procedures. Student's t tests were used to evaluate the difference between the simplified RNA isolation method and the traditional RNA extraction procedure in regards to the operation time, RNA concentration, A260/280 ratio, A260/230 ratio, RIN value, 28 s/18 s ratio, and Ct values of the ACTB, GAPDH and RPL13A genes. The level of statistical significance was set at $P < 0.05$.

3 Results

A total of 23 MSCs-HAP scaffold composites were constructed (Fig. 1). Nine (39.1%) of them were seeded with MSCs of sample #1, 7 (30.4%) with MSCs of sample #2,

and the remaining 7 (30.4%) with MSCs of sample #3. Among the 23 MSCs-HAp scaffold composites, RNA from 10 composites (43.5%) was isolated using simplified methods, while the remaining 13 (56.5%) were used to extract RNA according to traditional procedures. No significant differences existed among the three human MSCs sources regarding the number of scaffolds used for RNA isolation by the two different methods (Table 2). The average operation time required in the simplified RNA isolation steps (38.9 ± 0.5 min, range, 38.3 to 39.5) was significantly shorter than that needed in the traditional RNA

extraction procedures (79.9 ± 1.0 min, range, 78.8 to 81.1) ($t = 123.802$, $P < 0.001$) (Fig. 4).

3.1 Evaluation of RNA Concentration, Purity and Integrity

NanoDrop analysis results showed that the concentration of RNA extracted by the simplified methods was superior to that obtained by the traditional procedures ($t = 7.486$, $P < 0.001$) (Fig. 4). Our simplified RNA isolation procedures yielded RNA with superior purity compared to the traditional RNA extraction steps according to the A260/280 ratio ($t = 11.603$, $P < 0.001$) and A260/230 ratio ($t = 3.122$, $P = 0.005$) (Fig. 4).

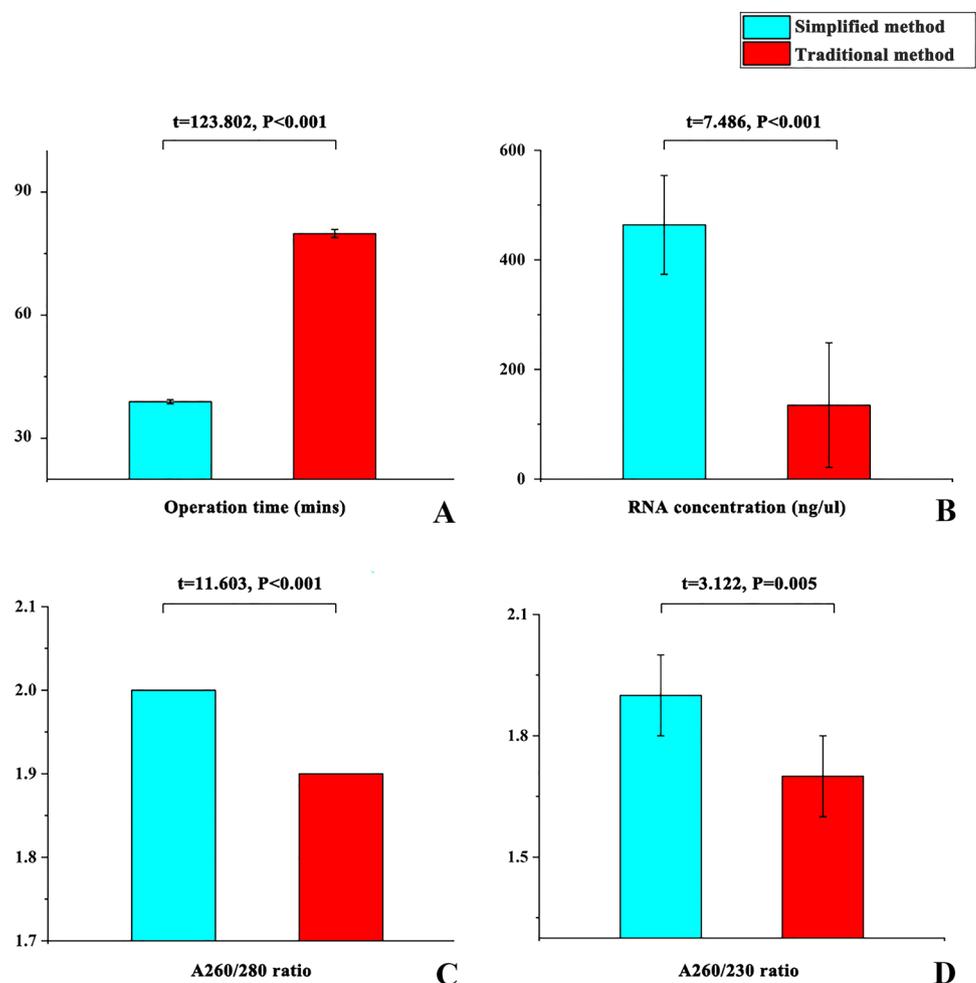
The Agilent 2100 Bioanalyzer analysis results indicated that the average RIN value and 28 s/18 s ratios of RNA samples obtained by the simplified method were 9.1 ± 0.2 (range, 8.9 to 9.4) and 1.3 ± 0.1 (range, 1.2 to 1.4), respectively (Fig. 5).

Table 2 Comparison of the number of MSCs-HAp scaffolds used for RNA isolation by two different methods among three human MSCs samples

	Sample #1	Sample #2	Sample #3	F	P
Simplified method	4	3	3	Fisher	1.000
Traditional method	5	4	4		

MSCs mesenchymal stem cells, HAp hydroxyapatite

Fig. 4 The comparison between two different RNA isolation methods in regards to operation time (A), RNA concentration (B), A260/280 ratio (C) and A260/230 ratio (D)



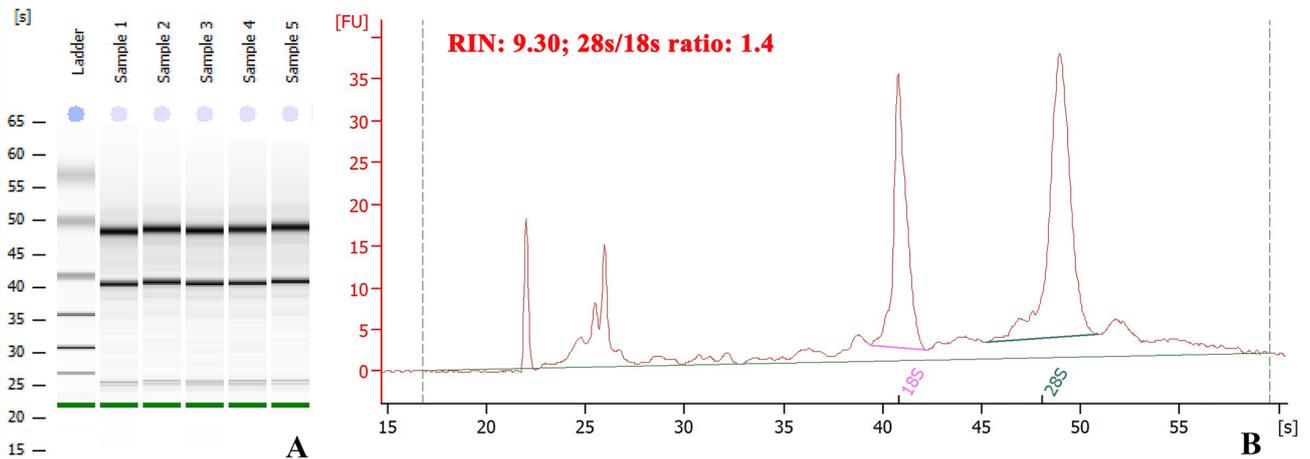


Fig. 5 The integrity of RNA yielded by the simplified isolation method. **A** Gel-like image of RNA sample running; **B** electropherogram of RNA peaks of sample run

3.2 Identification of Appropriate Reference Gene

The results of qRT-PCR analysis indicated that the mean Ct values of ACTB, GAPDH, and RPL13A genes in RNA samples obtained using the simplified RNA isolation method were significantly lower than those in RNA samples extracted by the traditional procedures ($P < 0.05$). Detailed data are shown in Fig. 6.

The Ct values of the GAPDH gene were significantly inferior to those of the ACTB and RPL13A genes in RNA samples obtained by the simplified procedures ($F = 455.15$, $P < 0.001$) or traditional steps ($F = 228.61$, $P < 0.001$) (Fig. 6). One-way ANOVA showed that the mean Δ Ct values were similar among ACTB (0.4 ± 0.4), GAPDH (0.5 ± 0.3) and RPL13A (0.3 ± 0.2) genes in RNA samples obtained using the simplified RNA isolation methods ($F = 0.719$,

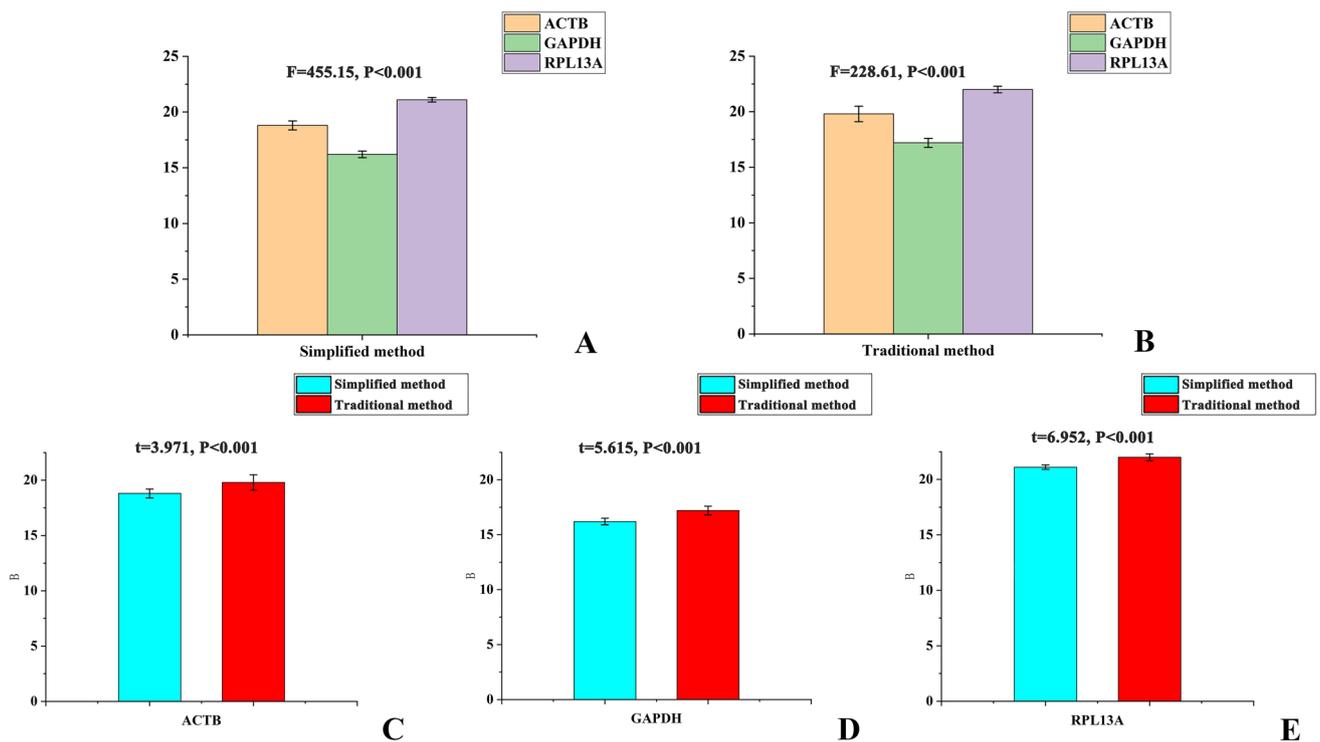


Fig. 6 Comparison of the Ct values of ACTB (**A, B, C**), GAPDH (**A, B, D**) and RPL13A (**A, B, E**) genes in RNA samples obtained by the simplified (**A**) or traditional (**B**) methods

$P=0.497$); similar results were also found in RNA samples isolated according to the traditional procedures ($F=2.482$, $P=0.105$) with ΔC_t values of 0.9 ± 0.7 , 0.5 ± 0.4 and 0.5 ± 0.3 for ACTB, GAPDH and RPL13A genes, respectively. Regardless of the isolation methods (simplified or traditional), the SD of ΔC_t values (0.4, 0.7) of ACTB was larger than those of GAPDH (0.3, 0.4) or RPL13A (0.2, 0.3).

4 Discussion

Our current study found that the simplified RNA isolation method was superior to the traditional single-step procedure regarding the operation time, RNA quantity, and A260/280 and A260/230 ratios in RNA extraction from MSCs-HAp scaffold composites. The RIN values and 28 s/18 s ratio of the yield RNA based on the simplified method showed excellent integrity. The C_t values and the SD of ΔC_t values between several genes indicated that GAPDH was the most appropriate reference gene in the MSCs-HAp scaffold composites.

The present study showed that the simplified tissue homogenization procedure was validated to extract RNA from MSCs-HAp scaffolds and showed superiority to traditional procedures in terms of operation time. This improvement is based on the specific structure of the MSCs-HAp scaffold, which is fragile, porous and embedded with less extracellular matrix [12, 13]; therefore, the HAp scaffold can be easily cut into large fragments, and the RNA extraction solution allows infiltration into the inside of scaffold fragments through an easy vortex procedure. As a result, the traditional homogenization procedures, including homogenizing the scaffold into powder using hammer hitting or ultrasound concussion, were unnecessary. In addition, the quantity of RNA obtained from the simplified methods is superior to that obtained by the traditional procedures. This difference could be explained by the fact that in our simplified homogenization procedures, the HAp scaffold cutting and vortexing steps could be finished in the RNA extraction solution in one container, which could save time and minimize sample loss. In contrast, traditional homogenization procedures require multiple containers to transfer RNA, and the time-consuming steps lead to RNA degradation [7]. In addition, the inferior RNA quantity in the traditional procedure may be attributed to the larger exposure of HAp powder to RNA when compared to large HAp fragments. Several studies have reported that RNA can be absorbed by HAp [10, 11]. In our simplified method, the large HAp fragment and vortex procedure may minimize RNA absorbance by the HAp.

The results of our study indicated that a column-based purification method might isolate RNA with superior purity when compared to the traditional method according to the

A260/280 and A260/230 ratios. Our result was in accordance with previous studies reported by Cepollaro et al. who used column-based purification methods to purify RNA from eight bone tissue samples and obtained RNA with an average A260/280 ratio of 2.04 and an average A260/230 ratio of 2.11 [20]. This superiority to the traditional method could be attributed to the specific structure of the RNA filtration column, which can absorb RNA and allow contaminants to pass through during centrifugation. However, in the traditional method, the RNA purification procedure was completed by centrifuging the RNA solution and then removing the supernatant detergent, which could not be fully removed, especially in the bottom of the container. In addition, the operation time required in our simplified RNA purification steps was shorter than that of traditional procedures. This result could be partially explained by the easy absorption of RNA by the filtration column, while long centrifugation times are needed to isolate RNA from the solution in traditional procedures [7]. In addition to the superior purity, the column-based purification method did not destroy the RNA integrity according to the average RIN (9.1 ± 0.2) and 28 s/18 s ratio (1.3 ± 0.1).

Notably, the C_t values of reference genes in the RNA isolated with our simplified methods were higher than those in RNA samples isolated by the traditional methods. This difference could be attributed to the fact that the traditional methods yield two kinds of RNA (long RNA and short RNA), while our simplified methods only yield long RNA due to the utilization of a specific RNA purification column that can only absorb long RNA [7, 14, 15]. Therefore, within the same volume, the quantities of reference genes that existed in the long RNA in the samples containing the long and short RNA were lower when compared to those in RNA samples only including the long RNA. Despite the single quality of RNA, long or short, obtained by our column-based method, it was possible to obtain RNA with superior quality and purity; the method can also be applied to short RNA studies provided that a column designed to absorb short RNA is utilized.

Additionally, our current results showed that GAPDH was the most appropriate gene for normalization in gene expression studies by comparing the C_t values and the SD of ΔC_t values among three commonly used reference genes (ACTB, GAPDH, and RPL13A). Our results agreed with previous studies that focused on MSCs-HAp scaffold composites [21–23]. In the studies reported by Ma et al., GAPDH was used as the reference gene in the gene expression analysis of the MSCs-HAp scaffold composite [21]. Zhang et al. and Salvadè et al. also reported similar results [22, 23]. However, these studies could not provide any information about why other reference genes were not selected in their reports [21–23]. To our knowledge, this study was the first to compare three commonly used

reference genes in the MSCs-HAP scaffold composite and identified GAPDH as the most appropriate gene that could be used in further analysis.

It should be noted that there are some limitations to the present study. First, comparisons with other RNA isolation methods were not performed. However, our simplified RNA isolation method was first validated to be convenient and efficient for extracting intact RNA in high quantities and could be used in other tissue engineering fields. Second, whether an RNA purification column that absorbs short RNA could be applied to the simplified method was not evaluated. Despite these limitations, our study is the first to report that RNA purification columns can obtain long RNA with good quality and purity from MSCs-HAP scaffold composites.

5 Conclusion

The simplified method using convenient homogenization procedures and column-based purification steps was time-efficient and validated for the isolation of RNA with superior quantity, purity and integrity from MSCs-HAP scaffold composites when compared to the traditional single-step RNA isolation methods. Finally, GAPDH was the most appropriate reference gene for further gene expression analysis.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [QW, WW and MG]. The first draft of the manuscript was written by [QW, WW and MG], other authors including [PZ, ZZ, TL, JL and FC] helped the data collection and statistical analysis. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Sun Yat-sen University (no.2019110401).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent to Publish None.

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