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Modifications in routine protocol of RNA isolation can improve quality of RNA purified from adipocytes

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ABSTRACT

Adipose tissue is of interest in the context of its role in the pathogenesis of cardiovascular diseases. Modern experimental techniques require a well-purified RNA, but all the routine protocols for RNA extraction have a number of limitations in case of fatty tissues. Here we described a modified protocol for RNA extraction from human adipocytes based on routine column method. Suggested modifications optimized the sample preparation, lysis and washing lead to enhance RNA purity. We conclude that the current protocol for total RNA purification from adipocytes allows extracting a high-quality RNA devoid of fatty acids, organic solvents and salts contamination.

Adipose tissue (AT) has traditionally been viewed as a fat depot and a source of energy, but the accumulation of knowledge about secretory and endocrine functions of adipocytes is leading to change of the present paradigm and recognition of AT role in regulation of energy homeostasis, reproductive function, immunity and cardiovascular system functioning [1–3]. AT is of interest in the context of its role in pathogenesis of type II diabetes, cardiovascular diseases (hypertension, atherosclerosis), endothelial and myocardial dysfunction and dyslipidemia [1,4]. It is known that visceral (VAT) and subcutaneous adipose tissue (SCAT) produce unique adipokines [3]; the volume and secretome of VAT correlates positively with cardiovascular disease incidence [3,5–7]. In the last decades AT has became the focus of interest for many researchers and is used in different studies.

The high quality RNA is needed for multiple techniques, including sequencing, reverse-transcription quantitative PCR, microarrays etc. In fact, it is challenging to extract a well purified RNA from fatty tissues like adipose or brain due to their high content of fatty acids and relatively low amount of other molecules like nucleic acids and proteins. Moreover, the number of cells in such tissues is usually very low that leads to poor yield of RNA. All routine protocols for RNA extraction have a number of limitations when used for fatty tissues, including RNA contamination by salts/fat/organic substances, loss of RNA during extraction, and RNA degradation [8–10]. The low quality of RNA is a big challenge principally in conditions of limited amount of material (e.g. biopsies of perivascular [PVAT] and epicardial adipose tissue [EAT] from patients with cardiovascular diseases), when contamination of extracted RNA may interfere with downstream application, and low yield of RNA will not allow repeating an experiment. The accuracy and reliability of results are the other challenges. In case of AT, in addition to adipocytes the biopsy may have traces of other cells like preadipocytes, endothelial cells, fibroblasts and various immune cells [11], so it is important to eliminate other contaminating cell types prior to extraction.

In this work we described the modified protocol of RNA extraction from adipocytes optimized for high-quality RNA purification from adipocytes in conditions of limited amounts of starting material compared to the routine protocols (TRIzol™ Reagent and RNeasy® Plus Universal Mini Kit).

14 samples of SCAT, PVAT and EAT were used in this study. Tissues were obtained from patients of Kemerovo Cardiology Center (Kemerovo, Russia) undergoing coronary artery bypass grafting, placed in transport medium (Hank's Balanced Salt Solution [Sigma-Aldrich, USA] + 100 U/L penicillin +100 mg/mL streptomycin +50 μg/mL gentamycin) and immediately transferred to the laboratory for adipocytes isolation. Adipocytes isolation was performed according to accepted recommendations given by K.A. Carswell [12]. After isolation the floated fraction of adipocytes was transferred to a new tube with culture medium and number of cells was scored.

Harvested cells were immediately processed to RNA purification. RNA purification from isolated adipocytes was performed using routine TRIzol™ Reagent (Invitrogen, USA) and RNeasy® Plus Universal Mini Kit (Qiagen, Germany) protocols, as well as the modified protocol that was

Abbreviations: AT, Adipose tissue; VAT, Visceral adipose tissue; SCAT, Subcutaneous adipose tissue; PVAT, Perivascular adipose tissue; EAT, Epicardial adipose tissue

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developed in our laboratory. The volume of cell suspension from 300 μL (SCAT) to 100 μL (PVAT, EAT) were used for total RNA purification using all mentioned methods (total number of cells in each sample was not more than 5×10^5). In case of TRIzol™ Reagent and RNeasy® Plus Universal Mini Kit we followed by protocols suggested by manufactures. Our modified protocol was based on the combined protocol for total RNA purification from AT described by S. Cirera [10] (inasmuch as this protocol is the most suitable for fatty samples out of the current published ones) with some modification. We describe our protocol step-by-step below (effective not modified steps from the routine RNeasy® Plus Universal Mini Kit protocol are marked with an asterisk):

1. Centrifuge cells suspension at 50 × *g* at room temperature for 1 min. Very carefully transfer supernatant containing adipocytes into a clean 1.5 mL tube.
   Note: It is important to avoid the damage of adipocytes in this step, because it can lead to poor yield of RNA.
2. Add 0.3 mL of cold 1 × PBS to the tube to wash the sample. Gently resuspend cells in PBS and centrifuge cells suspension at 50 × *g* at room temperature for 1 min. Very carefully transfer supernatant into a clean 1.5 mL tube. Repeat this procedure twice.
   Note: It is important to avoid the damage of adipocytes in this step, because it can lead to poor yield of RNA.

3a. *Add 0.75 mL QIAzol™ Reagent (Qiagen, Germany) per 0.25 mL sample (3:1 ratio) to the supernatant.
3b. Thoroughly homogenize the sample using syringe with 21G needle by pipetting it up and down.
   Note: It is important to achieve the complete homogenization to complete lysis of adipocytes.
4a. Incubate the homogenate for 8 min at room temperature.
4b. Centrifuge the sample at 12000 × *g* at 4 °C for 10 min.
4c. Completely discard the upper fat monolayer with a micropipettor. Transfer the clean supernatant (without disturbing the pellet containing cells debris) to a new 1.5 mL tube.
   Note: It is important to avoid the traces of fat in the final sample, because it can interfere with the purity of extracted RNA.

5. *Add 0.1 mL of gDNA Eliminator Solution (Qiagen, Germany) supplied with RNeasy® Plus Universal Mini Kit per 1 mL of QIAzol™ Reagent (1:10 ratio) and vortex it thoroughly.
6. *Add 0.2 mL of chloroform per 1 mL of QIAzol™ Reagent (1:5 ratio) and vortex it thoroughly.
7. Incubate the mixture for 6 min at room temperature.
8. Centrifuge the sample at 12000 × *g* at 4 °C for 30 min.
9. *Carefully transfer the clean upper aqueous phase without disturbing of interphase containing proteins or red organic layer.
   Note: The traces of proteins and organic substances/salts in the final sample can interfere with the purity of extracted RNA.
10. Add 1.5 vol of 95% ethanol and mix thoroughly by pipetting up and down (do not vortex).
11. Transfer up to 0.7 mL of sample into an RNeasy® Mini spin column supplied with RNeasy® Plus Universal Mini Kit placed in a 2 mL collection tube. Centrifuge the column at 10000 × *g* at room temperature for 15 s. Discard the flow-through and reuse the collection tube in step 12.
12. Repeat step 11 using the remainder of the sample. Reuse the collection tube in step 13.
13. Add 0.7 mL of RWT buffer (supplied with RNeasy® Plus Universal Mini Kit) to the RNeasy® Mini spin column. Centrifuge the column at 10000 × *g* at room temperature for 15 s to wash the membrane. Discard the flow-through and reuse the collection tube in step 14.
14. Add 0.5 mL of RPE buffer (supplied with RNeasy® Plus Universal Mini Kit) to the RNeasy Mini spin column. Centrifuge the column at 10000 × *g* at room temperature for 15 s to wash the membrane. Discard the flow-through and reuse the collection tube in step 15.
15. Add 0.5 mL of RPE buffer to the RNeasy® Mini spin column. Centrifuge the column at 10000 × *g* at room temperature for 2 min to wash the membrane. Discard old collection tube with the flow-through.

16a. Place RNeasy® Mini spin column in a new 2 mL collection tube and centrifuge it at full speed at room temperature for 2 min. Reuse the collection tube in step 16b.
16b. Add 0.5 mL of 70% ethanol to the RNeasy® Mini spin column. Centrifuge the column at 10000 × *g* at room temperature for 15 s to wash the membrane. Discard the flow-through and reuse the collection tube in step 16c.
16c. Add 0.5 mL of 70% ethanol to the RNeasy® Mini spin column. Centrifuge the column at 10000 × *g* at room temperature for 2 min to wash the membrane. Discard old collection tube with the flow-through.
16d. Place RNeasy® Mini spin column in a new 2 mL collection tube and centrifuge it at full speed at room temperature for 2 min. Discard old collection tube with the flow-through.

17. Place RNeasy® Mini spin column in a clean 1.5 mL microcentrifuge tube. Add 0.03 mL of RNase-free water directly to the spin column membrane and elute the RNA by centrifugation at 10000 × *g* at room temperature for 1 min.
18. Repeat step 17 using the eluate from step 17 and the same 1.5 mL tube.

Quantity and quality of extracted RNA were evaluated using Thermo Scientific™ NanoDrop 2000 Spectrophotometer (USA) by measuring the absorbance at 260 nm (A260) for determination of the concentration of RNA; at 280 nm (A280) and 230 nm (A230), as well as calculation of 260/280 ($A_{260/280}$) and 260/230 ($A_{260/230}$) ratios for assessment of the purity of RNA. Integrity of the extracted RNA was determined by electrophoresis in 3% agarose gel with the following visualization using Gel Doc™ XR + System (Bio-Rad Laboratories, Inc., USA).

Statistical analysis was performed using the software StatSoft STATISTICA 7.0. Mean (M) and Standard Deviation (SD) were calculated, Mann-Whitney *U* test was used to determine the significant differences between groups.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The results of total RNA purification from isolated adipocytes are presented in Table 1. The highest concentration of RNA

Table 1
Yield and quality of the total RNA extracted from adipocytes.

Sample	Tissue source	Volume of cells suspension, μL	Protocol	RNA yield (ng/μL)	260/280 ratio	260/230 ratio
1	SCAT	300	TRIzol™ Reagent	77.2	1.62	0.49
2	VAT	100	TRIzol™ Reagent	29.0	1.73	0.36
3	VAT	100	TRIzol™ Reagent	30.5	1.72	0.34
4	SCAT	300	TRIzol™ Reagent	38.4	1.68	0.45
5	VAT	100	RNeasy® Kit	14.2	1.99	0.93
6	VAT	100	RNeasy® Kit	26.8	1.88	1.20
7	VAT	100	RNeasy® Kit	13.5	1.85	1.01
8	VAT	100	RNeasy® Kit	16.5	1.86	1.16
9	VAT	100	Combined	31.6	2.12	1.92
10	SCAT	300	Combined	85.4	2.10	1.87
11	VAT	100	Combined	18.3	2.07	1.89
12	VAT	100	Combined	26.8	2.10	2.02
13	VAT	100	Combined	25.3	2.08	2.04
14	VAT	100	Combined	30.5	2.07	1.94

Abbreviations: SCAT – subcutaneous adipose tissue, VAT – visceral adipose tissue.

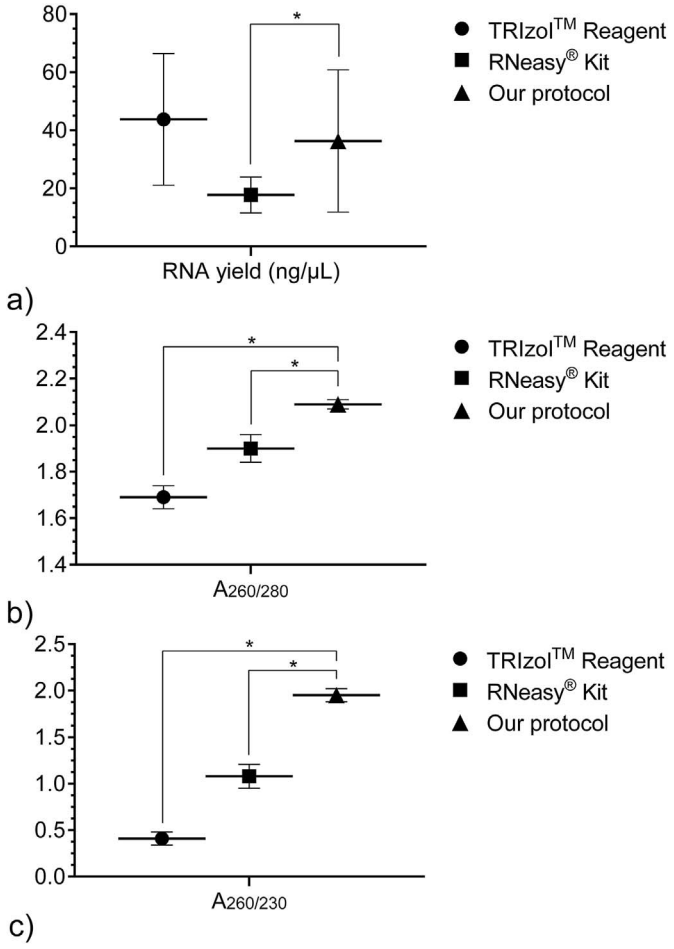

Fig. 1. The comparison of RNA yields (A), $A_{260/280}$ (B) and $A_{260/230}$ (C) in samples extracted by different protocols (μ ± SD).
Significant differences: $^{*}P < .05$ (Mann-Whitney *U* test).

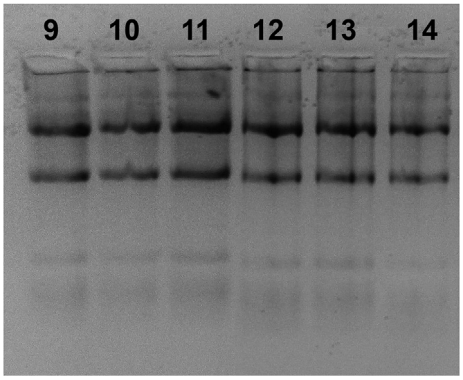

Fig. 2. Gel electrophoresis of RNA samples extracted by modified combined protocol.

(43.78 ± 22.66 ng/μL) was detected in the samples extracted by TRIzol™ Reagent method. At the same time, the purity of RNA was insufficient – $A_{260/280}$ and $A_{260/230}$ ratios were 1.69 ± 0.05 (compared to the optimal value of 2.1 for pure RNA) and 0.41 ± 0.07 (optimal value is 1.8 or greater), respectively. These results reveal the presence of proteins, traces of organic solvents (phenol/chloroform) and salts (guanidine isothiocyanate) that remain in the samples of RNA extracted by phenol-based method [10]. In addition to these substances, RNA extracted from adipocytes may contain such specific pollutants like fatty acids and polysaccharides of growth media, that also can affect the purity of RNA and lead to a decrease in 260/230 ratio.

In case of RNeasy® Plus Universal Mini Kit we extracted purer RNA ($A_{260/280}$ and $A_{260/230}$ ratios were 1.90 ± 0.06 and 1.08 ± 0.13, respectively), but these results were still insufficient, and there was a loss of RNA during extraction by column method (the mean concentration of RNA extracted by RNeasy® Kit in our experiment was 17.75 ± 6.17 ng/μL). It should be noticed that the purity of RNA extracted using these two routine protocols in our experiments was similar to results presented by other researchers [8,10].

The mean RNA yield (36.32 ± 24.50 ng/μL) and purity (2.09 ± 0.02 for the $A_{260/280}$ ratio and 1.95 ± 0.07 for the $A_{260/230}$ ratio) extracted by our modified protocol were significantly ($P < .05$) increased compared to RNA isolated by routine methods (Fig. 1). We have also showed no degradation and gDNA contamination in the RNA samples extracted by our modified protocol (Fig. 2).

The high RNA yield and purity achieved by our modified protocol were the outcome of optimization of sample preparation, lysis and washing steps. As it was mentioned, we added three washing steps before lysis of the sample to avoid any traces of growth media containing organic substances and polysaccharides that can interfere with the purity of extracted RNA. Considering the adipocytes are collected in a floated fraction in supernatant that also contains traces of growth media, it is important to remove this contaminant from samples. In our experiments we established that three washing steps are sufficient to completely remove residual growth media. After each centrifugation step, the supernatant containing adipocytes floats above the mix of PBS and growth media. Accurate collection of this floated fraction followed by adding another volume of PBS, resuspension and centrifugation leads to complete purification of samples from organic substances and polysaccharides. The increased the incubation time in steps 4a and 7 from 5 to 8 and from 3 to 6 min suggested in our modified protocol positively affects to RNA yield as this provides a more complete lysis and dissociation of the nucleoprotein complexes, which is important in the conditions of small amount of starting biological material. Additional steps of column washing by 70% ethanol before elution of RNA positively affect to the purity of final RNA sample, because this modification allow to completely avoid traces of fatty acids, organic substances and other contaminants presented in samples.

As it was shown in our experiments, the certain modifications of routine column protocol of RNA isolation from biological samples with high fat contents allowed getting purer total RNA compared to other methods. Moreover, the protocol described in the present article is optimized for RNA purification from adipocytes in conditions of limited amounts of material.

Conflict of interest

The authors have no conflicts of interest.

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