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Rapid and Cost-Effective RNA Extraction of Rat Pancreatic Tissue

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TITLE:

Rapid and Cost-Effective RNA Extraction of Rat Pancreatic Tissue

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KEYWORDS:

Purity, Extraction, RNA, Pancreas, Autolysis, Cost- effective

SUMMARY:

The purity and integrity of the isolated RNA is a vital step in RNA dependent assays. Here, we present a practical, rapid, and inexpensive method to extract RNA from a small quantity of undamaged pancreatic tissue.

ABSTRACT:

Regardless of the extraction method, optimized RNA extraction of tissues and cell lines are carried out in four stages: 1) homogenization, 2) effective denaturation of proteins from RNA, 3) ribonuclease inactivation, and 4) removal of contamination from DNA, proteins, and carbohydrates. However, it is very laborious to maintain the integrity of RNA when there are high levels of RNase in the tissue. Spontaneous autolysis makes it very difficult to extract RNA from pancreatic tissue without damaging it. Thus, a practical RNA extraction method is needed to maintain the integrity of pancreatic tissues during the extraction process. An experimental and comparative study of existing protocols was carried out by obtaining 20-30 mg of rat pancreatic tissues in less than 2 minutes and extracting the RNA. The results were assessed by electrophoresis. The experiments were carried out three times for generalization of the results. Immersing pancreatic tissue in RNA stabilization reagent at -80 °C for 24 h yielded high

integrity RNA, when the RNA extraction reagent was used as the reagent. The results obtained were comparable to the results obtained from commercial kits with spin column bindings.

INTRODUCTION:

Structural gene data can be transcribed to a functional product through gene expression. RNA analysis is used to discover differences in gene expression across different conditions. There are a number of methods to extract nucleic acids as follows: guanidinium thiocyanate, extraction via phenol-chloroform, cellulose-based chromatography, extraction by silica matrices, and anion-exchange[1,2].

Proper detection of gene expression is influenced by the integrity of RNA isolated from tissues; therefore, it is vital to evaluate the integrity of RNA isolated from tissues before further tests are carried out because complementary molecular tests on low-quality RNA may jeopardize diagnostic application results. Thus, high integrity RNA is needed for molecular biological tests with different diagnostic applications: quantitative RT-PCR, micro-arrays, ribonuclease protection assay, northern blot analysis, RNA mapping, and cDNA library construction[3,4].

RNA becomes rather unstable after being kept for a long time. Long mRNA fragments over 10 kb are particularly susceptible to degradation[5,6]. Thus, researchers must consider various factors that influence the integrity of purified RNA. The purity of RNA must be protected against RNases, proteins, genomic DNA, and enzymatic inhibitor contamination. In addition, the best and acceptable absorption ratio of RNA to UV (260/280) must be within the range of 1.8-2.0 with minimum fragmentation over electrophoresis. Recently developed laboratory techniques have enabled scientists to evaluate the integrity of molecular analysis sample more practically[7,8].

It is much more difficult to extract undamaged RNA from pancreatic tissue than other types of tissues because of the high quantity of ribonucleases (RNases). However, existing extraction methods, namely the rapid ejection of the pancreatic tissue from the abdominal cavity and homogenization at low temperatures to impede RNases, have proven ineffective[7-14].

The purpose of the present comparative experimental study is to modify and compare existing methods to determine the most efficient methods. To that end, various protocols of RNA extraction were modified and compared. It was specifically aimed at determining the least expensive method requiring a minimum amount of pancreatic tissue.

PROTOCOL:

Ethical approval for this study was obtained from Shiraz University of Medical Sciences (Approval number: 93-01-01-7178\03-07-2014).

NOTE: Use male Sprague–Dawley rats weighing 250 g. Place the vial containing a sliver of pancreatic tissue immersed in RNA stabilizing reagent in a liquid nitrogen tank at -80 °C and use RNA extraction reagent solution to maintain the integrity of RNA.

1. Removal of the rat pancreatic tissue

1.1. Prepare the operating room and place all required materials under the hood.

1.2. Sterilize all the surgical instruments (Dressing forceps, Brown-Adson forceps, Iris scissors, and Littler scissors) in an oven for at least 4 h at 240 °C to inactivate RNases[15]. Sterilize the surface of the surgery place with 70% alcohol under the hood.

1.3. Inject ketamine/xylazine intraperitoneally using an insulin syringe [80/8 mg/kg]. Check the depth of anesthesia by pinching the rat's toes for a lack of response.

1.4. Add 1 mL of RNA stabilization reagent in the proper microtube (2 mL) to inhibit activation of endonucleases in excised tissues.

1.5. Immediately place the rat on the surgical board (head away from the surgeon) for a mid-line incision.

1.6. Sterilize the entire abdominal surface with 70% alcohol and remove rat hair using a hair clipper with #40 blades.

1.7. Make a V-shape incision to open the abdomen from the pubic area to the front legs with Dressing forceps, Brown-Adson forceps, Iris scissors, and Littler scissors.

1.8. Flip the abdominal organs to the left side to expose the pancreas. Carefully find the pancreatic tissue, which spreads in the abdominal cavity. Locate the area under the spleen to find the pancreas without being confused with the fat tissue.

1.9. Immediately remove 20-30 mg of pancreas from the abdominal cavity in less than 2 min. Put the removed tissue in the 2 mL microtube quickly and cut it with a sterile cutter to penetrate the separated tissues with RNA stabilization reagent.

1.10. Place the microtube in a nitrogen tank to freeze immediately. Keep the microtube in a -80 °C freezer for 24 h[16].

1.11. After 24 h, transfer the tiny pieces of pancreatic tissues to an ice container.

1.12. Inject potassium chloride (KCl) intracardially for euthanasia of rats after surgery.

2. RNA extraction

2.1. Sterilize the surface under the hood with 70% alcohol.

2.2. Put 1 mL of the RNA extraction reagent, which contains guanidinium thiocyanate to inhibit RNase, in the sterile microtube.

2.3. Transfer the separated pancreatic tissue to the microtube. Transfer the microtube to the liquid nitrogen to freeze immediately.

2.4. Homogenize the tissue with the micro tip probe sonicator at 4 °C set to level 20 for 60

s on and 5 s off.

2.5. Incubate each homogenized sample for 5 min at 4 °C using crushed ice to allow the nucleoprotein complexes to be completely dissociated.

2.6. Enrich the samples with 0.2 mL of chloroform for each 1 mL of RNA extraction reagent. Cap the tube firmly and shake it forcefully for 15 s.

2.7. Incubate the tube for 15 min on the crushed ice (4 °C) to separate the reagent in three phases.

2.8. Run the centrifuge at 12,000 x *g* for 15 min at +2 to +8 °C. Transfer the colorless aqueous phase to a new microtube.

2.9. Add 0.5 mL of 100% isopropanol to the aqueous phase. Close the tube and then invert it at least 3 times to mix the RNA thoroughly.

2.10. Incubate the sample for 5-10 min on a cold box (4 °C) to allow RNA precipitation.

2.11. Run the centrifuge at 12,000 x *g* for 15 min at +2 to +8 °C. Discard the supernatant.

2.12. Enrich each of the centrifuge tubes with 1 mL of 75% ethanol.

2.13. Wash the RNA pellet by inverting the tube at least 3 times.

2.14. Run the centrifuge at 7,500 x *g* at +2 to +8 °C for 5 min. Discard the supernatant to remove the excess ethanol from the RNA pellet by air-drying.

2.15. Re-suspend the RNA pellet in diethylpyrocarbonate (DEPC)-treated RNase-free water.

2.16. Pass the solution through a pipette tip several times to dissolve the RNA pellet. Then, incubate the solution for 10-15 min at +65 °C.

3. Evaluating RNA Integrity with denaturation electrophoresis

3.1. Prepare gel running buffer: 50 mM NaAc (DEPC treated) and 0.5 M EDTA (pH 8.0) in DEPC-treated H_2O.

3.2. Prepare 5x formaldehyde gel-running buffer (MOPS running buffer): 0.1 M MOPS (pH 7.0), 40 mM NaAc, 5 mM EDTA (pH 8.0).

3.2.1. Dissolve 20.6 g of MOPS in 800 mL of DEPC treated 50 mM sodium acetate. Adjust the pH to 7.0 with 2 N NaOH. Add 10 mL of DEPC-treated 0.5 M EDTA (pH 8.0). Adjust the volume of the solution to 1 L with DEPC-treated water.

3.2.2. Filter the solution through a 0.2 µm filter and keep it at room temperature away from light for the sake of sterilization. The buffer becomes yellow with time if it is exposed to light or is autoclaved. A straw-colored buffer works well, but darker ones do not[17].

3.3. Prepare a 1.5% agarose gel[18]. For 50 mL, add 0.75 g of agarose and 31 mL of H_2O. Microwave the solution in the microwave for 1 min. Add 9 mL of formaldehyde and 10 mL of 5x MOPS running buffer.

3.4. Prepare the samples for the gel. Mix the following in a sterile microfuge tube:
X µL RNA (up to 30 µg)
2 µL of 5x gel-loading buffer
10 µL of formamide
4 µL of MOPS running buffer
1 µL of 0.1 mg/mL EtBr
3 µL of formaldehyde 5-x µL of DEPC-H_2O

3.5. Incubate the samples at 65 °C for 15 min and cool them on ice. Centrifuge for 5 s until all the fluid is deposited.

3.6. Pre-run the gel at 5 V/cm for 5 minutes.

3.7. Load the samples into the lanes of the gel immediately and then submerge the gel in 1x formaldehyde gel-running buffer. Run at 3-4 V/cm[19].

REPRESENTATIVE RESULTS:

Evaluation of the integrity of RNA in the RNA extraction reagent according to a routine and modified surgical protocol without RNA stabilization reagent

Unacceptable bands were observed after the extraction of RNA with the RNA extraction reagent from a routine surgical protocol. Lane 1 shows RNA from the liver as a control. Lane 2 shows the degraded status of 28S/18S rRNA bands in total RNA obtained from a routine surgical protocol. When the quantity of pancreatic tissue was reduced to 50 mg (lane 3) or 20-30 mg (lane 4) and the surgery was performed immediately (modified protocol) without the RNA stabilization reagent, RNA separation was less successful than in the liver tissue control and unspecific bands were observed.

Evaluation of the integrity of RNA samples according to a modified surgical protocol immersed in RNA stabilization reagent

The integrity of RNAs produced with the RNA extraction reagent depends on the preservation time and temperature (lane 5-8). In comparison with the control liver tissue, RNA separation was not successful when the amount of pancreatic tissue was 50 mg (lane 5) or 20-30 mg (lane 6). RNA was extracted immediately after the tissue was immersed in RNA stabilization reagent. No specific band was observed when 20-30 mg of tissue was submerged in RNA stabilization reagent at -80 °C for 48 h and RNA was extracted based on the protocol. According to the electrophoresis results, the RNA was completely degraded (lane 7). As depicted in lane 8, acceptable bands (28S/18S rRNA) were observed after submerging 20-30 mg of pancreatic tissue in RNA stabilization reagent at -80 °C for 24 h, and then RNA was extracted.

FIGURE AND TABLE LEGENDS:

Figure 1: Assessment of the integrity of RNA isolated from rat pancreatic tissues using RNA extraction reagent according to the protocols under the investigation

Lane 1 depicts the integrity of RNA obtained from the liver as a control. Lane 2 represents the status of 28S/18S rRNA bands in total RNA obtained from a routine surgical protocol. Lane 3 represents the status of 28S/18S rRNA bands in total RNA obtained from a modified surgical protocol and 50 mg of tissue. Lane 4 represents the status of 28S/18S rRNA bands in total RNA obtained from a modified surgical protocol and 20-30 mg of tissue. Lane 5 represents the status of 28S/18S rRNA bands in total RNA obtained from a modified surgical protocol and 50 mg of tissue extracted immediately after being immersed in RNA stabilization reagent. Lane 6 shows the integrity of RNA obtained from 20-30 mg of pancreatic tissue from a modified surgical protocol extracted immediately after being immersed in RNA stabilization reagent. Lane 7 depicts the integrity of RNA obtained from 20-30 mg of tissue from a modified surgical protocol after 48 h of immersion in an RNA stabilization reagent at -80 °C. Lane 8 depicts the integrity of RNA obtained from 20-30 mg of tissue from a modified surgical protocol after 24 h of immersion in an RNA stabilization reagent at -80 °C.

DISCUSSION:

In molecular biology it is vital to obtain high-quality RNA. The presence of the ribonuclease enzymes in cells and tissues quickly degrades RNA and makes the extraction complex. RNases are stable enzymes functioning without any co-factors. Small amounts of RNase are adequate to destroy RNA. When the rat pancreatic tissue is removed from the abdominal cavity, it is necessary to disinfect the surgical instruments by strong detergents, rinse them thoroughly and put them in an oven for at least 4 h at 240 °C to inactivate RNases before surgery. Given the fact that the RNase level is extremely high in the pancreas, the place of surgery is sterilized with NaOH and mild bleach to deactivate the RNases. While pancreatic tissue is being removed during dissection, the RNA would degrade. To increase efficiency, it is necessary for the dissection to be completed as quickly as possible[20-24].

The pancreas is a critical tissue for the body's homeostatic mechanisms. Hence, improved pancreatic RNA extraction procedures help researchers better understand active pathways. The present protocol proposed a model for an efficient, simple, and optimized method for RNA extraction from the pancreas. Different common RNA extraction methods from pancreatic tissue were evaluated. It was concentrated on the effect of frozen storage and RNase inhibition strategies influencing RNA quality. The two most significant factors influencing the integrity of RNA are surgery duration and the amount of collected pancreatic tissue. Recent studies have revealed that there is a positive correlation between RNA degradation and the amount of pancreatic tissue[8,13,20].

In this protocol, 20-30 mg of pancreatic tissue was obtained in less than 2 min from the anesthetized rats. Lengthy surgical steps may lead to activation of endogenous endonucleases in the pancreas and degrade the RNA quickly. In this study, RNA was isolated from different samples with guanidinium thiocyanate, and phenol-chloroform extraction techniques used liquid nitrogen to impede RNA activity. The method achieved three objectives: rapid permeation of RNA stabilization reagent in pancreatic tissues, protection of cellular RNA, and increased preservation time. The results were optimal when the samples containing RNA stabilization reagent were kept at -80 °C for 24 h.

However, RNA integrity increased significantly when RNA stabilization reagent was introduced. Moreover, this process was reproducible. A small section of the pancreas (20-30 mg) was dissected during surgery from anesthetized rats and submerged in 1 mL of RNA stabilization reagent at -80°C for 1-2 days. As seen in lane 8, storage for 24 h was the optimal time.

The aforementioned methods allowed a smaller quantity of RNA stabilization reagent to penetrate into the organ. Furthermore, the degradation process discontinued shortly after the experiments because the size of the pieces dissected was small. In this protocol, the vital step is cutting the immersed tissue in RNA stabilization reagent to very small pieces as soon as possible until it penetrates the cells and suppresses activation of RNase. In the homogenizing step, it is very crucial to prevent bubble production in the RNA extraction reagent and perform all steps at 4 °C. It is crucial to separate the aqua phase (the phase containing RNA) very carefully to avoid DNA contamination. Although autolysis and the presence of endogenous RNases compromise intact RNA isolation from the rat pancreas, the integrity of RNA was maintained in the proposed pancreas perfusion method. Thus, the proposed method is a straightforward, reproducible, and inexpensive procedure that requires smaller quantities of RNA stabilization reagent than the other existing methods.

Like any study, this protocol has some limitations. First, the integrity and yield of obtained RNA is less than using whole pancreatic tissue as only 20-30 mg of tissue is used. Second, a large number of samples cannot be taken in one day because RNA extraction and also cDNA synthesis tests must be done fast and consecutively to decrease RNA degradation. Third, to perform research projects with different rat groups, it is essential to take exactly 20-30 mg of tissue from the same surgical area to decrease variation of data because rat pancreatic tissue diffuses completely in the peritoneal cavity.

To conclude, using RNA extraction reagent solution after RNA stabilization reagent perfusion is a good alternative to expensive and column-based RNA extraction kits.

ACKNOWLEDGEMENT:
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DISCLOSURES:
None declared.

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Figure

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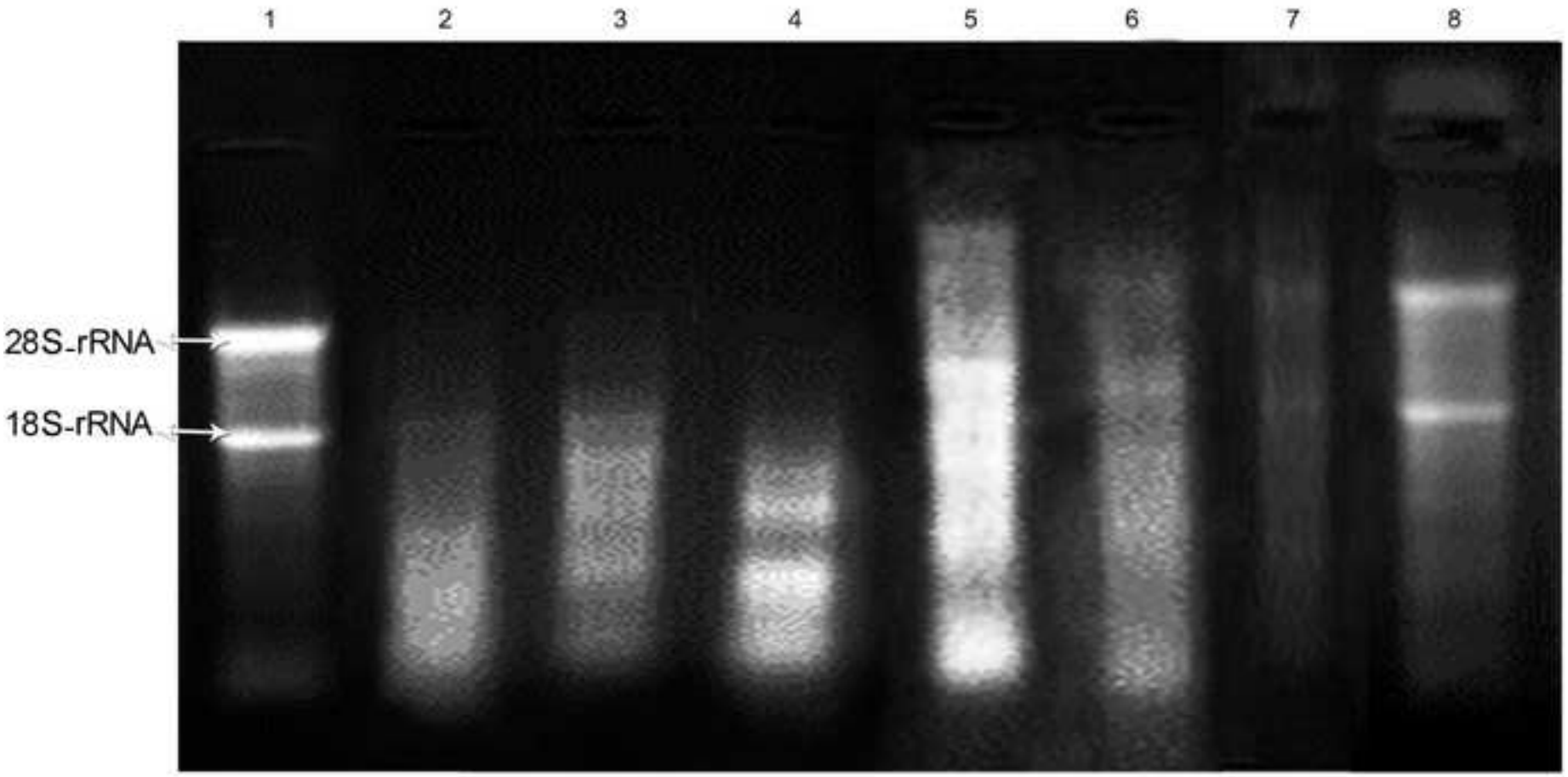

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agarose	Merck	116801	Germany
Atoclave	Teb Zaim		Iran
Centrifuge	Sigma		Germany
Chloroform	Merck	107024	Germany
Diethylpyrocarbonate (DEPC)-treated water	Sigma		Germany
EDTA	sigma	60-00-4	Germany
Electrophoresis tank	Payapajoohesh		Iran
Eppendorf microTube	Extragene		Taiwan
EtBr	sigma	E 8751	Germany
Ethanol	Merck	81870	Germany
Falcon Tube	Extragene		Taiwan
Formaldehyde	Merck	344198	Germany
Formamide	Merck	344206	Germany
Homogenizer-sunicator	Microson XL 2000		USA
Isopropanol	sigma	19516	Germany
Ketamine hydrochloride	sigma	1867-66-9	Germany
Laminar Flow Hood	Jal Tajhiz		Iran
Mgnetic stirrer	Labrotechnik		USA
Microcentrifuge	Eppendorf		Germany
Micropipette Tips	Extragene		Taiwan
MOPS	sigma	85022106	Germany
Na AC	Merck	567422	Germany
NaOH	Merck	109137	Germany
Oven	Teb Zaim		Iran
PH meter	Knick		Germany
RNA Later/RNA stabilization reagent	Qiagen	76104	USA
Surgical instrument	Agn Thos		German made
Syringes	AvaPezeshk		Iran
TriPure reagent/RNA extraction reagent	Roche	11667157001	USA
Vortex	Labinco		Netherland
Water bath	Memmert		Germany

zylazine	sigma	7361-61-7	Germany

August 2, 2020

Dear Editor-in-Chief

The authors would like to take this opportunity to express their appreciation to the editor and the reviewers for their constructive suggestions and recommendations for improving the manuscript. The detailed feedback notes on different aspects of the paper by reviewers have made the paper the best it could be. All of the reviewers' comments were considered and the manuscript has been revised accordingly. The responses of the specific comments of the reviewers are listed in this letter. Revisions in the text are shown using yellow highlight for modifications.

We have tried to consider your points. I hope you find the revision satisfactory otherwise we would appreciate if you could give us more explanation.

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thank you for your thoughtful comment by the respected Editor. The manuscript was thoroughly proofread according to spelling or grammar issues to ensure about its relevant and correct.

2. Figure 1: Please include a space between the number and the unit in Lane 1: 28S and 18S RNA. The S should be capitalized as well to represent the Svedberg.

Thank you for your thoughtful comment, the figure was corrected based on your valuable comment.

3. Please sort the Materials Table alphabetically by the name of the material.

Thank you for your thoughtful comment, Table of Materials was alphabetically sorted.

4. Please number the affiliations in order of appearance. Affiliation 3 comes before Affiliation 4 currently.

Thank you for reminding this point that we missed, the order of authors affiliation was corrected. Page.1

5. Please revise the title to be more specified. What is the modification of the method?

Thank you for your thoughtful comment by the respected Editor. The specified and proper title has been replaced as follows:

"**The rapid and cost-effective Method for RNA Extraction of Rat Pancreatic Tissue"**

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all

commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: RNA Later, TriPure, RNeasy, Millipore, etc.

Thank you for your thoughtful comment by the respected Editor. The specified and proper reagents have been replaced as follows:

-Tripure to "**RNA extraction reagent** "

-RNA-Later to "**RNA stabilization reagent**"

-RNeasy micro kit to "**commercial Kit with spin column binding capacity**"

7. Please place the numbered superscripted reference before the punctuation.

Thank you for your thoughtful comment by the respected Editor. This item was corrected in revised version of manuscript.

8. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

According to your constructive suggestion, the proper sentence has been added in page 4 as follows:

"This study was carried out on male Sprague–Dawley rats, weighting 250 g. Ethical approval for this study was obtained from Shiraz University of Medical Sciences (Approval number: 93-01-01-7178\03-07-2014)." Page 4, line 3.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Thank you for reminding this point that we missed, all incorrect words were deleted such as "could be," "should be," and "would be" throughout the Protocol and also included the safety section like using hood, etc.

10. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Thanks for your thoughtful comments, according to your constructive suggestion, protocol steps were modified, appropriately and also proper references were added to show how to perform the protocol action.

11. 1.1: How is the room prepared?

Thank you for your thoughtful comment by the respected Editor. Our point was to prepare the surgery place such as putting dissection mat, sterile gauze and all requirements for surgery. This section was corrected, appropriately Page 5, line 3&4.

12. 1.2: How is it done correctly?

Thanks for your thoughtful comments, according to your constructive suggestion, these sections were modified, appropriately as follows: Page 5,line 4-6.

1.2 Sterilize all surgery instruments (Dressing and Adson Brown Forceps, Iris and littler scissors) in an oven for at least 4 h at 240ºC to inactivate RNases

1.3 Sterilize the surface of surgery place with 70% alcohol under hood.

13. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Thanks for your thoughtful comments, according to your constructive suggestion, any personal pronouns were removed.

14. What animal is used? What is the age/gender/strain?
Thank you for reminding this point that we missed, this study was carried out on male Sprague–Dawley rats, weighting 250 g. Page 4, line2.

15. 1.8: Is there a step missing here? How are the inner organs accessed? What a cut made?

Thanks for your thoughtful comments, according to your constructive suggestion, "doing V-shape incision to open the abdomen from the pubic area to front legs" explain about how to cut the abdomen and find the pancreas properly. Page 5,line 14-15.

16. Please specify all surgical procedures and the instruments used throughout.

Thanks for your thoughtful comments, according to your constructive suggestion, the name of all surgical instruments were inserted such as Dressing and Adson Brown Forceps, Iris and littler scissors. Page 5, line 4-5.

17. 1.13: What is the size of the tube?

Thank you for reminding this point that we missed, "micro tube 2 cc were used". Page 5, line 21.

18. 2.2: Homogenize with what parameters?

Thanks for your thoughtful comments, according to your constructive suggestion, these sections were modified, appropriately as follows:

"Homogenize the tissue properly with the micro tip probe sonicator at 4 °C set at level 20 for 60 sec with 5 sec on-off interval." Page 6, line 1-2.

19. 2.3: How cold is it?
Thanks for your thoughtful comments, according to your constructive suggestion, temperature 4° c was provided by using grinded ice. Page 6,line 3-4.

20. 2.6: What temperature?

Thanks for your thoughtful comments, according to your constructive suggestion, temperature 4° c was added to the text. Page6, line 7.

21. 2.9: 100% isopropanol alcohol?

Thank you for reminding this point that we missed. “100% absolute Isopropanol was used.” Page 6, line 11.

22. How is DEPC treatment done?

Diethylpyrocarbonate (DEPC)-treated is a RNAse-free water which is used in RNA extraction protocol to inhibit RNase activity. In current study, prepared DEPC treated water was bought from Sigma Company.

For preparing this solution in laboratory, 0.1% v/v DEPC solution with water is made and incubated for 2 hours at 37 °C and then autoclaved at least 15 min.

23. 3.3: How is the melting done?

The melting process was done by keeping the flask containing agarose and buffer 1 min in microwave. Page 7, line 26-27.

24. Representative Results: There are too many sub-sections. Please remove the subheaders and combine the sections.

Thanks a lot for your valuable comments. The extra sub-headers were removed and the sections combined as much as possible. Page8, line 18-32.

25. Please discuss limitations of the protocol in the Discussion.

Thank you for reminding this point that we missed, the limitations of the method were added to the discussion section. Page 12, line 4-10.

26. Please complete the Acknowledgement section.

Thanks for your thoughtful comments, according to your constructive suggestion the Acknowledgement section was completed. Page12 ,line 15-18.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

Thank you for your thoughtful comment by the respected Editor. We tried to increase the homogeneity between the video and the written manuscript.

2. Please reshoot the video with a tripod so that the video is not so shaky. The focusing of the camera work needs to be improved. Please do not use a handheld camera.

Thank you for your constructive suggestion, unfortunately it is not possible for us to re-record the total part of video again due to Corona virus quarantine situation in our Country. We tried to improve the quality of video as much as possible according to our facilities. I hope you find the revision satisfactory otherwise we would appreciate if you could give us more explanation.

3. The audio needs to be cleaned up to remove a lot of the background noises and muffling.

Thank you for your thoughtful comment. We tried to to remove a lot of the background noises.

4. The additional details from the written protocol requested above must be included in the video narration as well.

Thank you for your thoughtful comment by the respected Editor. The additional details based on your comments was added to video narration.

5. There must be a discrete representative results section.

According to your constructive suggestion, a discrete representative results section was inserted in 08:47 section.

6. There must be a discrete conclusion section.

According to your constructive suggestion, a conclusion part was inserted in 09:59 section.

7. JoVE Video Format Standards
• Please insert a title card according to JoVE video requirements.

Thank you for your thoughtful comment, a title card was inserted to JoVE video in 00:02 section.

• Each section of the protocol needs a stand-alone chapter title card. See the JoVE video requirements for details.

According to your constructive suggestion, the proper chapter title card was inserted to the video as follows:

"00:58 The removal of rat pancreatic tissue

03:40 RNA extraction

08:08 Evaluating RNA integrity with denaturation electrophoresis

08:47 Representative results

09:59 Conclusion"

• This is an animal procedure, an institutional ethics card according to JoVE video standards is required to be placed after the introduction and before the protocol.

According to your constructive suggestion, an institutional ethics card according to JoVE video standards was placed in the proper part of the video in 00:50 section.

8. Narration Coverage & Performance:

• 01:33 Narration says: "Immediately place rodent on surgical board, head facing the surgeon for midline incision." *The video clip shows the rat's head away from the surgeon, contradicting the narration.

Thank you for reminding this point, the sentence was changed based on your comment.

• 04:06-04:30 Be sure to eliminate extra microphone sounds that can be isolated and deleted. There is a 'bump' and 'scratch' on the microphone here that can be easily cut out.

Thank you for your thoughtful comment, this part was re-recorded to eliminate extra microphone sounds.

• 04:47 Start the narration for this step here and eliminate the pipette preparation steps from 04:32-04:47. We don't need to see the pipette tip being fixed, we just need to see the TriPure Isolation Reagent going into the microtube on ice.

Thank you for your thoughtful comment, the extra part of pipette preparation was eliminated.

• 05:49-05:56 "Homogenize the tissue properly with homogenizer which will take few minutes." This audio clip has a lot of microphone scratches and bumps, so it should be probably be re-recorded. Consider also rewriting slightly: "Homogenize the tissue properly with *a* homogenizer, which will take *a* few minutes."

Thank you for your thoughtful comment, the extra part of homogenizing of the tissue was eliminated and re-recorded properly.

• 06:05 "Incubate each homogenized sample..." This clip is also very scratchy and will need to be re-recorded.

Thank you for your thoughtful comment, the extra parts were eliminated and re-recorded properly to decrease scratchy.

• 07:25-07:51 For segments without narration, be sure to cut out the parts of the narration audio that has no actual speaking. There are often extra noises that are heard during those parts that can be easily deleted.

Thank you for reminding this point, the extra parts were eliminated and re-recorded properly to decrease the extra noises of video.

• 08:37-08:50 You can delete the extra narration audio where nothing is spoken. There are just microphone bumping sounds.

Thank you for your thoughtful comment, the extra narration audio where nothing is spoken was deleted.

• 09:49 Microphone bump heard here

Thank you for reminding this point, this part was deleted in edited video.

9. Editing Style & Pacing:

• 01:48-02:20 You can probably abbreviate a lot of this segment of the initial incision and cut. Consider describing the aim of the incision and cut and then only show the most important parts of this incision.

Thank you for your thoughtful comment, this section was corrected.

• 02:56-03:22 Some of this can be deleted as well. The important part is moving the pancreatic tissue to the tube and then mincing it. The latter part of the mincing is probably redundant and can be eliminated until you get to the gentle inversion shaking @03:22.

Thank you for your thoughtful comment, the redundant parts were eliminated and showed the most important parts of the surgery and just mincing the tissue.

• 03:38-03:49 During this 10 seconds part we're only seeing the tube being removed from the liquid nitrogen but nothing is being said. If this is extra footage that is unimportant, consider removing it.

Thanks for your thoughtful comments, according to your constructive suggestion, the extra and redundant parts were removed.

• 04:06-04:30 We are seeing under the hood here but there is no narration describing what is important. Consider adding some narration that can accompiany these shots and focus our attention or eliminate these shots.

Thanks for your thoughtful comments, according to your constructive suggestion, some narration was added in this part.

• 05:46-05:58 Cut out the beginning of this shot and time it to match the duration of the narration regarding the homogenization.

Thanks for your thoughtful comments, according to your constructive suggestion, the beginning of this shot was cut out and matched the time and duration of the narration regarding the homogenization.

• 07:25-07:51 Some of this part without narration can probably be cut out.

Thank you for reminding this point, this part was deleted.

• 07:52-08:03 You can eliminate this part and start the narration and action right at 8:03, where the pipette is taking up the isoproponal.

Thank you for reminding this point, this part was eliminated.

• 09:16-09:38 Adding the 75% ethanol to the tubes- we probably only need to see the ethanol being added to one tube to understand this step.

Thank you for reminding this point, this part was eliminated.

• 10:11-10:40 Only show the best shots of the excess ethanol being removed for clarity and understanding of the viewers. We have too much here without narration and possible redundant information.

Thank you for reminding this point, the redundant part was removed for clarity and understanding of the viewers.

• 11:16 Please use a dissolve transition here. This edit point is a "jump cut" which are not allowed.

Thank you for reminding this point, the transition was dissolved.

Please upload a revised high-resolution file
here: https://www.dropbox.com/s/m0hbdv68qv6n7bp/prj7.mp4?dl=0

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript described a protocol for isolation of high quality RNA from rat pancreatic tissue. This is a good description of the technically methods but could be improved by addressing the following concerns:

Major Concerns:

1) Authors need to show representative results for the expression of pancreatic marker genes and housekeeping genes among different protocols by qRT-PCR or other methods.

Thanks for your thoughtful comments, unfortunately, no product was amplified for pancreatic marker and housekeeping genes expression among different inappropriate protocols by qRT-PCR due to low integrity and quality of RNAs which was not acceptable to synthesis cDNA.

The results of real-time PCR for Insulin, PPARγ and β-actin about our modified and confirmed protocol was published in two articles as follows:

1- "Dastgheib S, Irajie C, Assaei R, Koohpeima F, Mokarram P. **Optimization of RNA extraction from rat pancreatic tissue. Iranian journal of medical sciences.** 2014 May;39(3):282.

2-" Assaei R, Mokarram P, Dastghaib S, Darbandi S, Darbandi M, Zal F, Akmali M, Omrani GH. **Hypoglycemic effect of aquatic extract of Stevia in pancreas of diabetic rats: PPARγ-dependent regulation or antioxidant potential. Avicenna journal of medical biotechnology.** 2016 Apr;8(2):65.

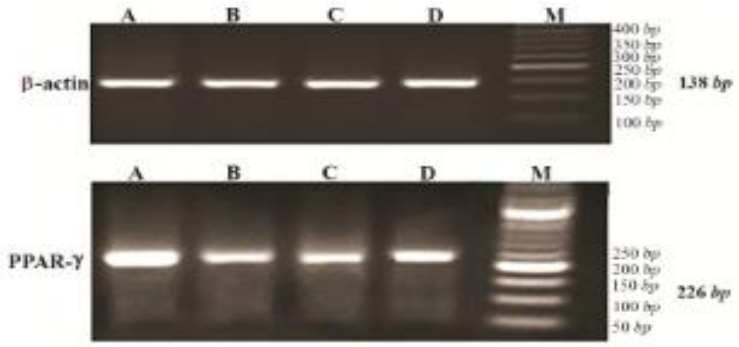

Figure 2. Agarose gel electrophoresis of RT-PCR products of PPARγ expression (226 *bp*) and β actin as internal control (138 *bp*) for experimental groups, stained with gel red. A) control group; B) diabetic group; C) diabetic rats received 10 *mg/kg* pioglitazone; D) diabetic rat received 400 *mg/kg* aquatic extract of stevia.

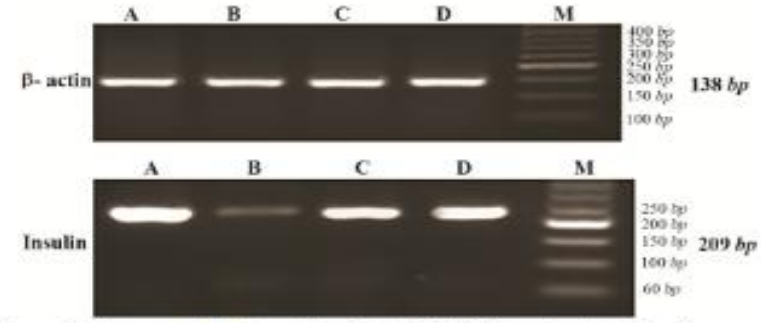

Figure 3. Agarose gel electrophoresis of RT-PCR products of insulin expression (209 *bp*) and β actin as internal control (138 *bp*) for experimental groups, stained with gel red. A) control group; B) diabetic group; C) diabetic rats received 10 *mg/kg* pioglitazone; D) diabetic rat received 400 *mg/kg* aquatic extract of stevia.

2) The RNA integrity of lane 7 and lane 8 were similar, but the bands of lanes 7 looked weaker. Was this due to the inconsistent sample load or the RNA yield was lower under the condition of lane 7.

Thanks for your thoughtful comments, these two lanes are related to the following conditions:

"Lane 7 depicts the integrity of RNA obtained from 20-30 mg tissue with modified surgery extracted after 48 h at -80ºC immersing in RNA stabilization reagent.

Lane 8 depicts the integrity of RNA obtained from 20-30 mg tissue with modified surgery extracted after 24 h at -80ºC immersing in RNA stabilization reagent."

It seems that the 28s,18s rRNA bands are just really acceptable in lane 8 not 7. As a matter of fact, the consistent amount of RNAs were loaded in each well and the reason of this discrepancy goes to the different integrity which is due to different extraction protocols.

3) A troubleshooting section is missing - this should be written to point out the common road blocks and how one should handle these.

Thank you for reminding this point that we missed, according the author guide the separate section for this subject was not found, so, the troubleshooting sections were added to the appropriate parts in conclusion. Page 11, line 26-31.

4) Authors need to carefully revise the manuscript to avoid misunderstanding by others.

a, In the figure legend, the authors did not describe the details of preservation time and temperature for lane 5 as lane 6-8.

b, The preservation time was 48 hours for lane 8 in the figure legend, however, the description in the representative results was 24 hours.

c, In the representative results, the author repeatedly described the results of lane 3-8.

d, On the line 197 the author says 50 mg tissue, but on line 200 says 100 mg tissue.

Thanks for your thoughtful comments, according to your constructive suggestion Page 10,line 12-20.

a) the using protocol in Lane 5-8 in detail were explained.
b) The details of preservation time and temperature for lane 5, thoroughly were corrected.
c) This part was also revised.
d) Thank you for reminding this point that we missed, this part was corrected.

Minor Concerns:

1) Is the method also applicable to the human pancreas?

Thanks for your thoughtful comments.

Isolating intact RNA is very demanding procedure since pancreas tissue contains high levels of RNase spontaneous autolysis. Main issues on this study is whether nature of murine pancreas has similar features of human pancreas, which is known as notorious samples for RNA study. Although, in current study, the protocol did not use in human pancreas, however, our protocol is probably applicable in the biopsy sample of patients which exactly taken in the time of surgery operation.

2) The author should describe the amount of tissue and corresponding RNA yield in each sample.

According to standard and reliable method, the amount of tissue in all lanes was described in current study. On the other hand, it is accepted that high yield of RNA doesn't show the high RNA integrity. For example, the yield of RNA in lane 6 and 8 were the same but as it is seen the integrity of them not comparable at all. Based on the literature the gold standard in evaluation of purity and quality of RNA is gel electrophoresis and the amount of 28/18/5 S rRNA is assessed. So, in current study, the integrity of RNA was compared not the yield.

Reviewer #2:

Manuscript Summary:

The manuscript tried to describe about efficient RNA isolation method from the pancreas of the rat using Tripure reagent. The protocol mentioned is a usual RNA isolation protocol generally followed by most of the scientists working on rat pancreas. There is no new step in the protocol, except collecting the tissue in 2 minutes.

The novelty of current study is comparing different protocols of RNA extraction and the best method with the highest RNA quality, was optimized.

In current study, a straightforward, rapid (less than 2 min), and cost-effective (just use 1cc RNA later) method was described for complete RNA extraction from the minimum amount (20-30 mg) of pancreatic tissue.

In previous protocols are recommended to inject the RNA-Later in vater ampulla (hepato pancreatic ampulla) to inactivate RNase, which the consumption amount of RNA-Later is too much.

We agree with this thoughtful comment by the respected reviewer. However, it is not possible for us to do this experiment because of the following reason:

Firstly, we tried to prepare the proper amount of RNA Later, for which we had to deal with a foreign company. Because of the new situation in Iran caused by international sanctions leading to limitations on currency transfer, it was very hard for us to prepare a suitable material.

The view of using the cost-effective method is very important for the researchers that live in Iran which accessing to the commercial materials is a challenging situation, furthermore that are so expensive.

Furthermore, as the full budget for this project has been paid by the Vice Chancellor for Research in our University, it was not enough to buy so expensive material, and it was not possible for them to pay any extra amount for this project.

In our optimized protocol, small amount of RNA later about 1 cc was consumed for each sample that was very cost-effective for us.

Reviewer #3:

Manuscript Summary:

In this manual script the authors described a protocol to extract RNA from rat pancreas, and preserve RNA integrity by 1. quick dissection. 2. immersing in RNA-later. 3. storage at -80 degrees for 24 hours.

Major Concerns:

This protocol does not add significant value to the literature. RNA-later is already used by many to preserve RNA. A simple Google search would yield several similar, if not more sophisticated, protocols.

https://www.pancreapedia.org/tools/methods/isolation-of-pancreatic-rna

https://www.sciencedirect.com/science/article/pii/S0003269716000695

https://www.future-science.com/doi/full/10.2144/0000113862

Thanks for this thoughtful comment by the respected reviewer. Although it is completely true about the use of RNA later in many protocols of RNA extraction of pancreatic tissue, however, using the cost-effective method is very important for us.

In previous protocols are recommended to inject the RNA-Later in vater ampulla (hepato pancreatic ampulla) to inactivate RNase, which the consumption amount of RNA-Later is too much. However, it is not possible for us to do this experiment because of the following reason:

Firstly, we tried to prepare the proper amount of RNA Later, for which we had to deal with a foreign company. Because of the new situation in Iran caused by international sanctions leading to limitations on currency transfer, it was very hard for us to prepare a suitable material.

The view of using the cost-effective method is very important for the researchers that live in Iran which accessing to the commercial materials is a challenging situation, furthermore that are so expensive.

In general, the full budget for our projects which is paid by the Vice Chancellor for Research in our University, is not enough to buy so expensive material. So, small amount of RNA later about 1 cc was consumed for each sample that was very cost-effective for us in our optimized protocol.

On the other hand, we published an article entitled "Optimization of RNA extraction from rat pancreatic tissue" in 2014 which is cited in several high impact factor journals such as PLOS ONE and other journals that are listed below. This article describes the best, cost-effective protocol.

We also published other article by using optimized method entitled "Hypoglycemic effect of aquatic extract of Stevia in pancreas of diabetic rats: PPARγ-dependent regulation or antioxidant potential" in 2016. In this study, the m RNA level of Insulin, PPARγ in 40 rats were evaluated and the results of all experiments was reproducible.

The following papers have been cited our work:

Choi S, Ray HE, Lai SH, Alwood JS, Globus RK. Preservation of multiple mammalian tissues to maximize science return from ground based and spaceflight experiments. PloS one. 2016 Dec 1;11(12):e0167391. Impact Factor: 2.806

Augereau C, Lemaigre FP, Jacquemin P. Extraction of high-quality RNA from pancreatic tissues for gene expression studies. Analytical Biochemistry. 2016 May 1;500:60-2. Impact Factor: 0.719

Yanni S, Lijing S, Shuqing L, Juan S, Jiejing C, Jun L. Effect of emodin on Aquaporin 5 expression in rats with sepsis-induced acute lung injury. Journal of Traditional Chinese Medicine. 2015 Dec 1;35(6):679-84. Impact Factor: 0.176

Cavicchioli L, Zappulli V, Beffagna G, Caliari D, Zanetti R, Nordio L, Mainenti M, Frezza F, Bonfante F, Patrono LV, Capua I. Histopathological and immunohistochemical study of exocrine and endocrine pancreatic lesions in avian influenza A experimentally infected turkeys showing evidence of pancreatic regeneration. Avian Pathology. 2015 Nov 2;44(6):498-508. Impact Factor: 2.338

siddique t, awan fr, najam ss, khan ar, anver j, qureshi mk, islam m, zain m. amplification, cloning and expression of the reg3 δ gene from mouse pancreas. pak. j. biotechnol. vol. 2015;12(1):55-61. impact factor: 0.192

Saccomandi P, Larocca ES, Rendina V, Schena E, D'Ambrosio R, Crescenzi A, Di Matteo FM, Silvestri S. Estimation of optical properties of neuroendocrine pancreas tumor with double-integrating-sphere system and inverse Monte Carlo model. Lasers in medical science. 2016 Aug 1;31(6):1041-50. Impact Factor: 2.299

Ghoraishi T. Strategies to combat inflammation in pancreas and islet transplantation.

Pławińska-Czarnak J, Zarzyńska J, Bogdan J, Majewska A, Karwański M, Kizerwetter-Świda M, Kaba J, Anusz K, Bagnicka E. An optimized method of RnA isolAtion fRom goAt milk somAtic cells foR tRAnscRiptomic AnAlysis. Annals of Animal Science. 2019 Jul 1;19(3):605-17. Impact Factor: 1.590

Khosrobakhsh F, Moloudi MR, Shoja K, Mohammadi S. Effect of Alpha-Lipoic acid on Pancreatic Optic Atrophy 1 (OPA1) Gene Expression in Male Rat Model of Obstructive Cholestasis and Cirrhosis. Scientific Journal of Kurdistan University of Medical Sciences. 2019 Dec 10;24(5):120-34. Impact Factor: 0.125

Khosrobakhsh F, Moloudi MR, Shoja K, Mohammadi S. Effect of Alpha-Lipoic acid on Pancreatic Optic Atrophy 1 (OPA1) Gene Expression in Male Rat Model of Obstructive Cholestasis and Cirrhosis. Scientific Journal of Kurdistan University of Medical Sciences. 2019 Dec 10;24(5):120-34. Impact Factor: 0.125

Minor Concerns:

-Copy-editing is very much needed for this manuscript.

-The authors should specify anesthetized for surgery or euthanized for dissection. If this is indeed a surgery, the authors should describe how to close wound and care for the animal(s) post-surgery.

-RNA gel electrophoresis is a common technique therefore does not need to be described in details.

-The manufacturer of TriPure reagent is Roche not ROCH.

Thanks for your thoughtful comments. Copy-editing was precisely done.

- Thanks a lot for your thoughtful opinion.

To our knowledge the anesthesia definition is more proper than euthanasia for this kind of research. Based on the findings, optimal collection strategy recommend that rodent tissues intended for metabolomics studies be collected under anesthesia rather than post-euthanasia.

Animals may be anesthetized for surgery, for non-surgical procedures that may be painful, or non-painful procedures that require immobilization. Anesthesia is the loss of feeling in all or part of the body, with or without loss of consciousness. So, combined ketamine/xylazine is the preferred injectable anesthetic in rats.

This work was a small part of the comprehensive research project that our team has been well done and our results was published. Our strategy was selected based on our research design. During collecting sample from pancreas, it is so important that animal should be alive. Activation of death phase exactly cause release of endonuclease enzymes and degradation of RNA in pancreas by onset of death.

However, several other tissues were also removed for further analysis and finally 10 cc blood was directly taken from the heart. So, once that the intact pancreas was removed, the animals were sacrificed for further investigation.

- Thanks for your thoughtful comments. RNA gel electrophoresis was summarized based on your comments.

- Thanks for your thoughtful comments. ROCH to ROCHE has been changed.

Reviewer #4:

This manuscript written by Sanaz Dastghaib et al is about Method for RNA Extraction of Rat Pancreatic Tissue. Isolating intact RNA is very demanding procedure since pancreas tissue contains high levels of RNase spontaneous autolysis. This study compares different protocols. This manuscript deals with clinically demanding subjects with good results. Manuscript was well written for complete RNA extraction from minimum quantitatively intact pancreatic tissue.

Main issues on this study is whether nature of murine pancreas has similar features of human pancreas, which is known as notorious samples for RNA study. It might be very useful that authors can try and get the same efficacy in human pancreas specimen. However even if it is not possible, this manuscript deserves to be published with minor editorial correction. If they want to try human experiment, they can refer the reference, in which Jun et al described the effective method in human pancreata. (Translational Oncology (2018) 11, Jun 800-807)

Thanks for your thoughtful comments by the respected reviewer. According to your constructive suggestion, the above article was cited in our manuscript.