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Research Article

ISOLATION OF RAT HEPATOCYTE USING COLD TRYPSINIZATION METHOD AND TOTAL RNA ISOLATION USING HOT SDS/PHENOL EXTRACTION METHOD

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ABSTRACT

Isolation of Rat hepatocyte for the purpose to access the metabolizing activity by cold trypsinization method is easy and cheap method to perform at small scale laboratories and academic research purpose. Kits are available for the isolation of eukaryotic RNA but cost related to the experimentation is generally very high and unaffordable to small budget laboratories. Hot SDS/phenol RNA isolation method is generally used for isolation of total RNA from E. coli. But by doing some modification in this method good quality of total RNA isolated from rat hepatocyte. Instant lysis of cells using hot sodium dodecyl sulphate (SDS) quickly inactivates endogenous RNases and hence this can be very important due to the extremely short half-life of many mRNAs and is of particular importance when performing time-course studies. Isolation of RNA using the hot SDS/Phenol extraction method is excellent and accurate method to get good quality of total RNA ($A_{260/280}$ ratio is 1.83), which can be further utilized in gene expression profile study.

Keywords: RNA isolation, Rat Hepatocyte isolation, Cold trypsinization, Hot SDS/Phenol extraction method, RNA Quantification

INTRODUCTION

In vitro metabolism from rat liver is primitive access to drug discovery in the research for right candidate drug during voyage of discovery of molecules. Liver is harbor of drug metabolizing enzyme systems which is subdivided in to two phases, I & II. Phase I metabolizing system detoxifies while phase II increases water solubility for clearance from excretory organ. During the metabolism, drug may induce or suppress the gene and may affect the drug metabolism or leads to drug-drug interaction[1] . RNA is a particularly labile bio-molecule and is much more susceptible to degradation by endogenous- and exogenous-nucleases and to non-specific degradation by divalent cations, heat, elevations in pH, and storage of tissue or cells over extended periods prior to RNA extractions, which result in falsely altered gene expression patterns[2]. Gene expression study is mainly carried out by isolation of RNA, cDNA formation is done by Reverse transcriptase and further amplification is carried out by DNA polymerases[3]. Several techniques and kits are being offered for RNA isolation, which have their own respective principle and methodology and are believed to perform equally well. Here two most reliable and widely used techniques for high throughput RNA isolation are described so briefly; 1) guanidine isothiocyanate-phenol: chloroform (GTC)-based RNA isolation technology and 2) Silica-gel column (SGC)-based RNA isolation technology. The GTC technique for isolation of RNA, which was developed by Chomczynski and Sacchi[4], is very popular because it requires much less time than other classical methods (e.g., $CsCl_2$ ultracentrifugation). Moreover, GTC salt denatures the cellular proteins and inactivates RNases ensuring that isolated RNA is not degraded. Many commercial reagents (e.g. Trizol®, RNAzol™, RNAwiz™) are based on this principle. While principle of SGC technology (Qiagen RNeasy Mini column) is a combination of the selective binding properties of a silica-based membrane with the speed of microspin technology, which allows saving time, money, and efficient use of small and precious biological samples. These RNA isolation techniques are very sensitive but expensive due to using very sensitive and precise chemical. Presence of abundant RNase in the atmosphere push to use sophisticated facilities. It needs to develop method which can be performed in the cost effective in case of procuring the chemicals and utility. Rat hepatocyte isolation by one step or two step collagenase perfusion technique, increase the cost due to presence of collagenase, which is costly than our crude trypsin.

Trypsinization is the method to disaggregate the tissue and isolate the cells from it using either warm or cold preexposure method. Due to tryptic activity of trypsin in warm trypsinization method at 37^0C; tissue gets damaged and harvesting of cells after 30 min incubation may lead to reaggregation or damage to cells. These demerits can be overcome by using minimum concentration of trypsin and long incubation time to increase the separation efficacy of trypsin. At longer incubation time the tryptic activity affects the cells and hence the temperature must be minimized to reduce tryptic effect. A simple method of minimizing damage to cells during exposure is to soak the tissue in trypsin at 4^0C for 6-18 h to allow penetration of enzyme with little tryptic activity followed by 20 to 30 min exposure at 37^0C for disaggregating[5].

Isolation of eukaryotic RNA can be done using readymade kits but cost related to the experimentation is generally very high and unaffordable to small budget laboratories. Hot SDS/phenol RNA extraction method is generally used for isolation of total RNA from *E. coli.*, but with some modification in the method isolation of good quality of total RNA from rat hepatocyte. Instant lysis of cells using hot SDS quickly inactivates endogenous RNases which is responsible for extremely short half-life of many mRNAs and which is of particular importance when performing time-course studies.

MATERIALS

3 g male wistar rat liver, Dulbecco's Modified Eagles Medium (DMEM), petridishes, scalpel blade, biosafety cabinet, Trypsin solution (0.25% in the DMEM media), SDS lysis solution (2% SDS in 16mM EDTA solution), Acid phenol/Chloroform solution (1:3), Chloroform/Isoamyl alcohol (1:3)

METHODS

Isolation of rat hepatocyte using trypsinization with cold exposure

Male wistar rat (250 g) was sacrificed by cervical dislocation and liver was isolated by making a horizontal incision on ventral side near to diaphragm[6]. Isolation time from the rat was not more than 2-3 min. Liver was kept in the Phosphate Buffer Saline (PBS) at 4^0C until used.

Experimental area was sterilized and all experimentation was done under biosafety cabinet. Liver was chopped by surgical blade in to small pieces of approximately 1 mm in size; these pieces were washed with PBS and transferred to other petridish and unwanted tissues such as fat and necrotic tissue were removed. Liver pieces were washed again with PBS; transferred to 50 ml vial and soaked in 0.25% crude trypsin in DMEM for 18 h at 4^0C. Trypsin was added in the ratio of 10 ml per 1 g of tissue. After incubation, pieces were exposed at 37^0C for 30 min and 1 ml of warm DMEM medium per

100 mg of tissue was added. To disperse the tissue completely, gentle pipetting was done in up and down fashion and filtered with sterile muslin cloth to remove the larger pieces. Cell viability was determined by hemocytometer using inverted fluorescent microscope and obtained 92.7%. Cells were preserved for repetitive seeding and stock culture by using 5% of Dimethyl Sulphoxide (DMSO) to total volume of cell culture as cryopreservative. Cells were seeded at 5 x 10^6 cells per ml into 75 mm^2 Tissue culture grade flask to grow the cells with 10% of Fetal Bovine Serum (FBS) and DMEM (with L-glutamine)[6].

Isolation of RNA from the rat hepatocyte

5ml of cells (8.2 x 10^6 cells per ml) were pipette out quickly and added directly into boiling lysis solution (Half volume of lysis solution was used relative to total volume of cell and media). The tube was kept at 100^0C for 5 min with periodic mixing. Mixture was poured directly into equal volume of 65^0C acid phenol/chloroform solution and tube was sealed with parafilm cap to avoid phenol escaping during mixing. It was then mixed well by frequent vortexing, kept at 65^0C for 10 min and centrifuged at 5000 RPM for 15 min. Aqueous phase was transferred by avoiding white interface to fresh 50 ml tube and an equal volume of acid phenol/chloroform was added. Cap was sealed with parafilm and mixed well by vortexing at high speed. Mixture was centrifuged at 5000 RPM for 15 min and aqueous phase was transferred to fresh 5 ml centrifuge tube. Equal volume of Chloroform/Isoamyl alcohol (1:3) was added to mixture, centrifuged at 7000 RPM for 15 min. Aqueous phase was transferred to 5 ml centrifuge tube and equal volume of isopropanol was added. This mixture was kept at -20^0C for overnight. Mixture was spin for 20 min at 14,000 RPM and at the bottom white pellet was observed. Supernatant was removed and pellet washed with 5 ml of ice cold 70% ethanol, dried at room temp ~20 min and reconstituted in 500 µl of autoclaved sterilized double distilled water. RNA sample was heated at 70^0C for 10 min to inactivate the DNAse. 50 µl of RNA sample was diluted ten times. Absorbance of RNA was measured at 260 and 280 nm[7].

Electrophoresis

The overall quality of an RNA preparation may be assessed by electrophoresis on a denaturing agarose gel which gives some information about RNA yield. A denaturing gel system is suggested because most RNA forms extensive secondary structure via intramolecular base pairing, and this prevents it from migrating strictly according to its size[8]. For total RNA quality determination, 2% of agarose gel was prepared by dissolving 2 g of agarose powder in 100 ml of 1X Tris-Borate-EDTA (TBE) buffer. The mix was cooked until get boiled, and then 10 µg/ml ethidium bromide was added, mixed, and poured in to gel electrophoresis unit. Each RNA samples (2 µg) were mixed with 5 µl of gel loading solution and were directly applied on a gel's wells. Horizontal electrophoresis was carried out at 100 V for 1.5 h in 1X TBE. The total RNA was assessed under gel documentation system. Horizontal gel electrophoresis was carried out using 2 µg of RNA sample in 2% agarose gel at 100 V for 1.5 h in 1X Tris-Borate EDTA (TBE) buffer. 10µg/ml of Ethidium Bromide was used as fluorescent dye to visualize single stranded RNA. Image was captured under UV mode using gel documentation system[9].

RNA quantification

50 µl of each RNA sample were diluted up to 450 µl of water (50 times dilution), pH 7.5 and measured in spectrophotometer at wavelength 260 and 280 nm.

RESULTS AND DISCUSSION

Absorbance of isolated RNA measured at 260 and 280 nm were found to be 0.4264 and 0.2314 respectively. $A_{260/280}$ ratio observed was 1.83 which shows good quality of isolated RNA. Literature data suggests 1 Absorbance of nucleic acid represent the 40 µg of RNA in sample prepared in water at pH 7.0

Volume of RNA sample = 5 ml

Dilution = 50 µl of RNA sample + 450 µl distilled water (1/10 dilution).

Measured absorbance of diluted sample in a 1 ml cuvette (RNase-free): A260 = 0.4264

Concentration of RNA sample = 40 x A260 x dilution factor

= 40 x 0.4264 x 10

= 170.56 µg/ml

Total yield = concentration x volume of sample in milliliters

= 170.56 µg /ml x 5 ml

= 852.3 µg = 0.85 mg RNA

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio of 1.8-2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Small changes in the pH of the solution will cause the 260/280 to vary. Acidic solutions will under-represent the 260/280 ratio by 0.2-0.3, while a basic solution will over-represent the ratio by 0.2-0.3[10]. $A_{260/280}$ ratio of 1.83 is observed and ratio of 1.83 indicates the good quality of RNA samples. Gel run shows the separation of RNA by two distinct bands[11](figure 1). Integrity of RNA is determined by the 18s and 28s Ribosomal RNA band during gel electrophoresis. Here in figure 1; lane 1 and 3 shows the two intact bands but lane 2 does not show second band. This result suggests the lane 2 sample is containing integrated RNA while lane 1 and 3 contains integrated RNA.

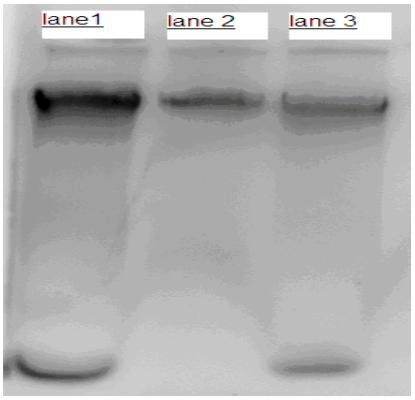

Fig. 1: Image of agarose gel using gel documentation system under UV mode

CONCLUSION

Rat hepatocytes are the excellent tissue/cell model to study the Pharmacokinetic or pharmacodynamic profile of various drugs and the metabolizing enzymes localized in liver. Cold trypsinization is proved to be an excellent method for isolation of rat hepatocytes and resulting primary cell culture can be used for further investigation on the drug action on the cell system based on the genotype-phenotype experimentation related to cell biology. Good quality and high yield of total RNA can be isolated by hot SDS/phenol extraction method, which has found to be highly efficient and economic for small scale laboratory purpose.

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