Original Article

Extraction of total RNA in the developing chicken forebrain

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Abstract

Background: Gene expression of Gama-Aminobutyric acid ($GABA_A$) receptor subunits may change during development. Procedures in molecular biology are required to understand the gene expression profile $GABA_AR$ in chicken. The outcome of the results depends on good-quality high-molecular-weight RNA. Several procedures can be used to isolate RNA from the brain of chicken; however, most of them are time-consuming and require disruption of cells or freeze and thaw in the presence of RNase inhibitors. The aim of this experiment was isolation of RNA from chicken embryonic brain tissues using appropriate RNA extraction kit.

Materials and Methods: Fertilized eggs from Ross breed (*Gallus gallus*) were incubated at 38°C and 60% relative humidity in a forced-draft incubator and were turned every 3 h. After 3, 7, 14 and 20 days of incubation, eggs were cooled on ice to induce deep anesthesia. Then whole brains were dissected out. As brains could not be excised in a reproducible way from earlier embryos (embryonic days 4 and 6), whole heads were collected. Chicken embryos between day 7 to 20 and 1 day after birth were decapitated, and their brains removed. Samples were immediately inserted into lysis buffer and stored at −70°C. Total RNA was isolated and a contaminating genomic deoxyribonucleic acid (DNA) was digested. RNA quality was checked using gel electrophoresis.

Results: We obtained 52 mg/ml to 745 mg/ml with A260/280 1.7-2.2. Only high-quality RNA, with no signs of degradation, was used for further experiments.

Conclusion: In conclusion, protocol was found to be suitable for the isolation of total RNA from embryonic chicken cells.

Key Words: Developing chicken forebrain, fertilized eggs, ribonucleic acid, extraction, ross breed

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INTRODUCTION

Gamma-Aminobutyric acid (GABA) is considered as the predominant inhibitory neurotransmitter in vertebrate central nervous systems (CNS). There are two major classes of GABA receptors: $GABA_ARs$ and $GABA_BRs$. The $GABA_A$ receptor belongs to the Cys-loop superfamily of ligand-gated ion channel and has a heteropolymeric structure that forms a chloride channel.[1,2] The $GABA_A$ receptor is derived from various subunits such as alpha1-alpha6, beta1-beta3, gamma1-gamma4, delta, epsilon, pi, and rho1-3.[2] Additional heterogeneity is produced by alternative splicing of some of the subunits. These subunits assemble as pentamers and similar to many other ion channels, their biophysical and pharmacological properties are dependent on the

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subunit stoichiometry.[3] Intensive research has been performed to understand and establish the distribution and functions of these receptors in the CNS. Expression-level profile of these receptors is not well-known in chick embryo during development. Most $GABA_A$ receptors in the mammalian CNS are thought to contain α, β, and γ subunits, with the most common receptor having a stoichiometry α1β2γ2.[3,4] High-potency benzodiazepine modulation of the $GABA_A$ receptor requires the presence of γ2 subunits.[4] Recent studies have suggested that among the various actions of benzodiazepines, receptors containing γ1 subunits are responsible for the sedative/hypnotic actions of benzodiazepines, whereas receptors containing γ2 subunits mediate their anxiolytic actions.[5]

Over the course of 20 days, a vast complex process of chick embryo development occurs.Previous studies have shown that during chick formation, parts of genes in chick may form and then disappear.[6]

The reason for this is not well understood yet, but it may probably be due to a cell process called apoptosis. Gene expression of $GABA_A$ receptor subunits may change during development. Because of this, there is much interest in understanding the gene expression profile of these receptors. Consequently, many experimental procedures in molecular biology are required for $GABA_AR$ in chicken. The outcome of the results depends on good-quality high-molecular-weight RNA.

Several methods are widely used to isolate RNA from chicken, including acid–phenol extractionand guanidinium isothiocyanate extraction.[7,8] Unfortunately, these methods are time-consuming, costly, and laborious. Several procedures can be used to isolate RNA from the brain of chicken; however, most of them are time-consuming and require disruption of cells or freeze and thaw in the presence of RNase inhibitors. These mechanical procedures incur the danger of mechanical and enzymatic degradation of the nucleic acids.

The purpose of this experiment was isolation of RNA from chicken embryonic brain tissues using appropriate RNA extraction kit.

MATERIALS AND METHODS

Much of the work done was in preparing the eggs for the experiment. Ross breed eggs were purchased from a commercial provider. The eggs were incubated under standard conditions and 24-h, 72-h, 7-day, 14-day, and 20-day eggs were collected for further processing. The next step was to examine them. Each embryonated egg shell was cracked and the contents were dropped off gently inside a sterile plate in a way that the blastoderm lies above the yolk sac. A filter paper ring was prepared and put around the blastoderm. The area opaca of the embryo was abstracted using forceps and scissors and placed in a sterile plate filled with Ringer's solution. Light microscopy was used to examine the presence of the embryos. The 72-h eggs were examined first.

Total cellular RNA was extracted from chicken brain tissues using an RNeasy® Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's directions. Briefly, recommended amounts of tissue (30 mg) were placed in the appropriate lysis buffer supplied with the kit (Buffer RLT and β-mercaptoethanol). At this point, the manufacturer's protocol was followed. The RNA was eluted twice in a total volume of 30 µl of RNase-free H_2O. Samples were immediately aliquoted and stored at −80°C. RNA was quantified by measuring A260 absorbance in a biophotometer (Ependorf, Germany). Purity was assessed by calculating the A260/A280 ratio.

RESULTS

The quantity and quality of RNAs isolated from the brain cells using the extraction method are summarized in Table 1. RNA extraction from brain cells produced 96 µg/ml of RNA at 24 h with an A260/280 ratio of 1.65 ± 0.15. RNA was also extracted from brain cells at other time intervals. The total yield of RNA was the highest in day 20 and the lowest in 24 h. The total yields ranged from 96 to 243 µg/ml from brain cells. This translated into an increase in yield of 1.4, 1.75, 1.8, and 2.5-fold for the 72 h, 7, 14, and 20 days, respectively, compared with 24 h. The total yields for the brain cells ranged from 96 to 243 µg/ml, again showing a substantial increase in RNA yield over that obtained using the RNeasy® procedure.

The RNA obtained from the brain cells were determined to be highly pure based on the measured A260/A280 ratios in the 1.90-1.95 range [Table 1]. Most samples also had 18S and 28S rRNA bands at approximately 1500-3000 bp [Figure 1]. The issue of RNA quality was assessed by calculating the A260/A280 ratio to determine DNA and protein contamination. Ratios

Table 1: Spectrophotometry results

Sample	Ratio 260/280	RNA (µg/ml)
24 h	1.65 (1.52-1.80)	96
72 h	2.04 (1.96-2.13)	136
7 day	1.73 (1.58-1.89)	168
14 day	1.55 (1.48-1.63)	172
20 day	1.48 (1.38-1.58)	243

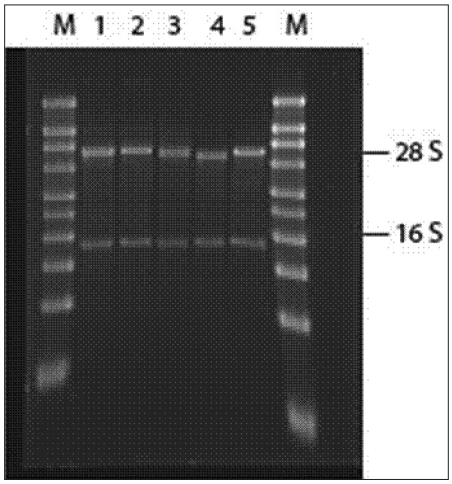

Figure 1: Agarose gel electrophoresis of RNA. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA samples (columns 1-5)

for all types of samples examined ranged from 1.48 to 2.04 [Table 1]. There were no significant differences among samples. The closest ratio to 2.0 (indicative of pure RNA) was obtained for 72 h RNA isolated through RNeasy® (2.04).The quality of RNA was inspected by agarose gel electrophoresis [Figure 1]. All samples showed bands typical of undegraded RNA.

Results of RNA extracted used for real-time polymerase chain reaction (RT-PCR) showed significant alterations of GABAA receptor gamma subunit genes in chicken embryo during development. This spatiotemporal expression pattern for GABA Areceptor gamma subunits in the forebrain can explain the diversity of GABAA receptor properties. Details of the results had been published in Iranian Journal of Basic Medical Sciences.[9]

DISCUSSION

Physiological and pharmacological features of GABA receptors may be changed during brain development. Therefore, studying these receptors is important for better understanding their physiological and pharmacological features. One way to approach this is determining the GABA receptor genes expression during brain development. Because of the special structure of the brain, it is not easy to gather suitable RNA. In this study, we have demonstrated that RNA can be consistently isolated from embryonic chicken brain tissues using a commercially available RNA extraction kit. The mRNA has been demonstrated to be of sufficient quality and quantity for successful use in downstream RT-PCR-based testing procedures including RT-PCR for gene expression.

Similar to reports in the literature, Qiagen RNeasy™ protocol did yield a proper amount of RNA from cells.[10] Therefore, the isolation kit that had previously been optimized for extraction of RNA from cells is also useful for extraction of RNA from chicken embryonic tissue. It is not surprising that the RNeasy Mini Plus™ protocol from Qiagen yielded very good detectable RNA as this procedure eliminates lower-molecular-weight RNA from the final isolated product.

It should be noted that small amounts of DNA were detected in the RNA specimens after isolation with the extraction kit. Complete DNA removal from RNA preparations is imperative for RT-PCR amplification of targets where the primary transcript lacks introns, thereby rendering amplification products derived from viral mRNA and contaminating DNA indistinguishable. Thus, additional DNase treatment of isolated RNA prior to RT-PCR was carried out in some of our studies.

The data presented in this study demonstrate that RNA of sufficient quantity and quality can be obtained from embryonic chicken cells and used reproducibly in applications such as RT-PCR.

We were able to isolate high-quality RNA successfully by grinding whole brain tissue. A key aspect to isolating clean RNA using Qiagen's RNeasy® was not to exceed the recommended amount of tissue by more than 20%. It is critical to stay within the recommended amounts (30 mg) if there is a high amount of mucus when working with this protocol.

Comparison of our RNA yields obtained from chicken brain cells with those from other extractions from brain is not possible due to the lack of such literature.

Absorbance ratios (A260/A280) between 1.8 and 2.0 can be used to determine the quality of an RNA preparation. The ratios reported for all samples in this study are slightly high. Brain tissue produced a single set of bands that are presumably the typical 28S and 18S bands of RNA.

The bench time required by our method of RNA extraction method RNeasy® was a significantly shorter protocol compared with other methods and this allows DNase treatment to be achieved on the column (without the DNase treatment, the protocol time is only 30 min).

CONCLUSION

This protocol was found to be suitable for the isolation of total RNA from embryonic chicken cells. If time is of concern, we recommend the use

of RNeasy® due to its simplicity. However, if large quantities of RNA are desired, it is advisable to use Trizol® because this method produces significantly larger amounts of RNA than the RNeasy® protocol and it is easily scalable.

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