COMPARISON OF THE EFFICACY OF RNALATER PRESERVED AND FRESH TISSUE IN GENE EXPRESSION STUDY OF FISH

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ABSTRACT

RNA isolation from tissues is an important methodology and a prerequisite for studying gene expression since all molecular biology methodologies and techniques do not occur at the same time. Furthermore, molecular biology research is heavily reliant on the use of stored samples. As a result, this study was conducted to compare the effect of RNALater stored fish tissue samples on isolated RNA integrity using the spectrophotometric method, and gene expression using quantitative RT-PCR. Clarias gariepinus tissues were collected and immersed in an RNAlater buffer before being stored at 4 °C for 14 days, at the inception of nucleic acid isolation, fresh tissue was included with the stored RNALater tissues. Results revealed that tissue storage in RNALater buffer has no effect on the purity and concentration of the RNA isolates. This study however shows that fresh tissue batch had a higher expression of the innate immune gene TNF-α, (i.e., of 0.411-fold expression higher) than the RNA-later batch (0.097). While RNALater effectively preserves RNA integrity in Clarias gariepinus tissues, it significantly alters TNF-α gene expression compared to fresh tissue (0.411-fold vs. 0.097-fold). This highlights the importance of careful storage method selection for accurate gene quantification studies.

Keywords: Gene expression, RNA quality, RNALater, A260/280 Absorbance, *Clarias gariepinus*

INTRODUCTION

Obtaining high-quality RNA is crucial for carrying out various molecular biology procedures, including microarray experiments and real-time RT-PCR (Die and Román, 2012). The level of salt, organic compounds, lipids, proteins, or DNA contamination in RNA samples depends on the tissue type, preservation, and purification techniques used during the experiments (Nouvel *et al*. 2021). Spectrophotometric methods can be used to detect impurities in the samples. Additionally, it is common knowledge that RNA can easily degrade if not handled with care (Tröβe *et al*., 2010).

In molecular biology, there are two popular techniques for preserving and storing RNA samples: flash freezing in liquid nitrogen and storing in aqueous sulphate salt solutions, like commercial RNAlater (Passow *et al*., 2019). Flash freezing, which involves immersing the sample in dry ice or liquid nitrogen, is the preferred method for stabilizing tissue samples for further analysis (Wolf, 2013). However, obtaining and transporting dry ice or liquid nitrogen can be challenging, especially in the field (Mutter *et al*., 2004). As a result, in the last ten years, many researchers, particularly in the field, have used RNAlater to store RNAseq-bound samples, which eliminates the need for immediate sample processing or tissue cooling. RNAlater can quickly penetrate tissue to stabilize and safeguard RNA (Chowdary *et al*., 2006; Florell *et al*., 2001).

RNA samples immersed in RNAlater can be safely stored at room temperature for up to a week, and even longer when kept at colder temperatures. Mutter *et al*. (2004) showed that tissue samples stored in RNAlater at room temperature for 72 hours yield accurate gene expression results compared to RNA samples isolated immediately after collection or 48 hours later in a gene expression study. RNAlater also allows for significantly longer storage times. This study investigated the effect of RNAlater buffer on RNA integrity for subsequent gene expression techniques compared to fresh fish tissue storage. Specifically, it aimed to answer two primary questions: The first being whether the tissue storage affects gene expression and secondly if the RNAlater buffer preserve the RNA integrity.

MATERIALS AND METHODS

RNALater Preparation / Sample Collection

To prepare the required stock solution for the reagents, a combination of 40 ml of 0.5 M EDTA, 25 ml of 1 M Sodium Citrate, 700 g of ammonium sulphate, and 935 ml of sterile distilled water was stirred on a hot plate stirrer over low heat until complete dissolution of ammonium sulphate. After

cooling, the pH was adjusted to 5.2 using 1 M H_2SO_4, and the solution was transferred to a screw-top bottle for refrigerated storage.

Tissue samples for the study were collected in two batches from cultured *Clarias gariepinus* species at the fish hatchery of the Nigerian Institute for Oceanography and Marine Research (NIOMR) Biotechnology department. In the first batch, samples (1 g) from the ventral region of *Clarias gariepinus* were collected in the Biotechnology molecular biology laboratory upon transport from the hatchery section. The tissue samples were immediately immersed in 1 ml of RNALater buffer, placed in well-labelled 2 ml Eppendorf tubes to preserve RNA stability, and stored at 4 ºC for 14 days until the initiation of RNA isolation. For the second batch, tissue samples were collected during the RNA isolation period.

RNA extraction and Reverse Transcription PCR

Following the storage of the initial sample batch in RNALater for 14 days, the second batch of fresh tissue samples was collected. Subsequent RNA extractions were carried out on both fresh and RNALater-stored samples utilizing the Zymo Plus Mini Kit. Total RNA quantification was performed using the NanoDrop Spectrophotometer from Thermo Fisher Scientific.

The synthesis of cDNA was accomplished through reverse transcriptase polymerase chain reaction (RT-PCR), and quantification was conducted using the housekeeping gene GAPDH along with the primers specified in Table 1.0.

Table 1: Primers used for Nucleic acid quantification and qPCR in this study

S/n	Primer	Forward Sequence	Reverse Sequence	Base Pair
1	GAPDH	GCCCTCTGGTAAAATGTGGA	ATTCCCTTCATGGGTCCTTC	450
2	β-actin	TGGCCGTGACCTGACTGAC	CCTGCTCAAAGTCAAGAGCGAC	180
3	TNF-α	GGATGGTGGTGTGTGTTGTG	CTGGTACTCTGGTCACGACTC	150

Comparative gene expression study

To compare the effect of RNALater buffer with fresh tissue on the expression of TNF-α, quantitative PCR was conducted. The 5x Hot Fire Pol Evergreen qPCR Supermix was used prepared according to the manufacturer's instructions. The qPCR profile steps for β-actin and TNF-α were performed as follows: initial denaturation at 95°C for 15 seconds (1 cycle), followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 1 minute, elongation at 72°C for 30 seconds, and final elongation at 72°C for 30 seconds. The CT values were exported using the quantitative PCR spreadsheet format. The relative quantification method was used to measure gene expression, employing the 2^-ΔΔCT method, as described by Schmittgen and Livak (2008).

RESULTS

RNA and cDNA quantification

To assess the potential influence of RNALater buffer on RNA quality during tissue preservation, the A260/280 absorption ratios were determined. Concentration and absorbance values of RNA isolates from both RNALater-stored and fresh tissue samples met the optimal concentration of 50 ng/µl. The average concentration and purity (1.8 to 2.1) of RNALater-stored tissue RNA adhered to the requirements for downstream applications, as indicated in Table 2, which details individual concentration and purity (Δ260/280) values for RNA isolates from RNALater-stored tissues (samples 1-5) and fresh tissues (samples 6-10). The quantified cDNA gel capture (Figure 1) illustrates the GAPDH PCR amplicons on a 2% agarose gel electrophoresis of reverse-transcribed RNA samples from both batches, commencing with RNALater-stored samples (wells 1-3) and progressing to fresh tissue samples (wells 4-6).

Table 2: Nucleic Acid quantification showing the Δ260/280 values and concentrations

in ng/µl of RNA Isolated from *Clarias gariepinus*

Sample (RNA)	Absorbance A260	Absorbance A280	Absorbance (A260/A280)	Concentration (ng/ul)
1	1.35	0.69	1.96	86.9
2	3.14	1.75	1.89	116.1
3	2.30	1.28	1.81	150.5
4	1.38	0.83	1.96	109.8
5	1.693	0.902	1.88	54.4
6	3.14	1.75	1.79	132.4
7	3.29	1.77	1.86	131.9
8	1.28	0.80	1.85	209.5
9	2.14	1.11	1.92	83.4
10	2.14	1.11	1.83	76.3

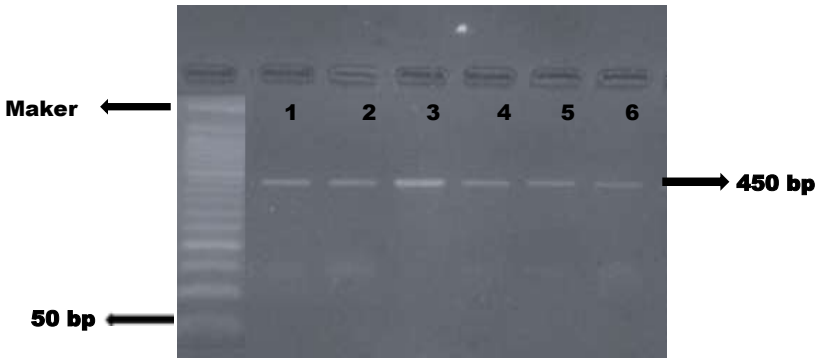

Figure 1: Gel image capture cDNA quantification of samples done in Triplicate from the two batches, GAPDH with expected amplicons band size of 450bp.

Quantitative real time-PCR

Figure 2 shows gene expression fold change of the gene of interest (TNF-α) we used in comparing the quantitative expression between the varying batches of RNA isolates in this study. The expression of the TNF-α gene was downregulated in all batches, with fresh tissue batch having a higher fold change of 0.411 compared with RNALater batch which had a lower mean fold change of 0.097.

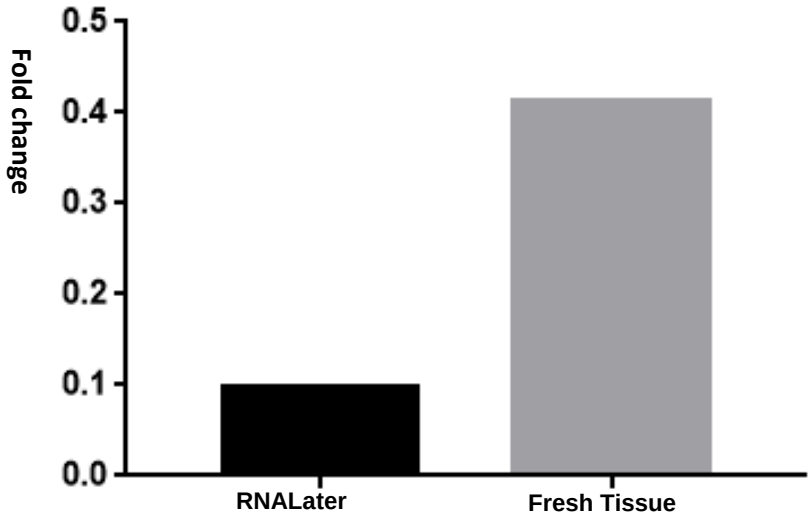

Figure 2: Gene expression Fold change of two batches used in this study.

DISCUSSION

In the present study we investigated and compared the possible effects of RNA preservation on RNA Isolates quality of *Clarias gariepinus* tissue stored in RNALater with fresh tissue. Furthermore, a comparative expression levels of a specific gene in Isolated RNA in the fish was also reported. The purity of the isolated tissue RNA from the two batches; RNALater stored tissue and fresh tissue, evaluated by the A260/280 absorption ratios both yielded pure RNA samples, though the mean absorption ratios were slightly higher for RNALater-preserved tissue, which had a mean absorption ratio of 1.9, indicating higher RNA purity (Held, 2008) than fresh tissue having absorption ratio of 1.85. The reverse transcribed RNA had visible GAPDH (housekeeping gene) PCR amplicons bands which also indicated success in cDNA synthesis and confirming the RNA absorption ratio result.

Having high-quality cDNA samples for downstream applications, the expression level of the TNF-α gene in *Clarias gariepinus* was measured by quantitative PCR. The chosen gene is an interesting candidate for use in this study on the molecular mechanisms of innate immunity and the proinflammatory response of fish to environmental pathogens, ultimately leading to a homeostatic response (Gao *et al*., 2012). The expression of TNF-α gene could thus be affected by environmental innate immune related response in *Clarias gariepinus*. In the present study, the quantitative expression of TNF-α was downregulated and significantly varied in all sampling batches, which is probably due to differences between the sampled fish which were in two batches (Jia *et al*., 2021). Expression levels of TNF-α was highest in Isolated RNA from the fresh tissue batch which had a fold change of 0.411 when compared to the RNALater batch containing the stored tissue with a fold change of 0.097. There was a non-significant variation in the concentration and purity of the isolated RNA, as well as the quantified cDNA of the two batches in this study as they all met the optimum values for downstream gene expression study (Chang-Hui, 2019). But there was a significant variation in quantitative TNF-α expression between the batches, where Isolated RNA from fresh tissue had a higher fold change value (expression) when compared with RNALater Isolates.

CONCLUSION

Based on the quantified results of the isolated RNA and cDNA, it can be concluded that tissue storage

does not significantly affect the concentration and purity required for downstream gene expression studies in fish tissues (*Clarias gariepinus*). However, this study did reveal a significant variation in the expression of a gene of interest, TNF-α, which may be attributed to the homeostatic status of the fish at the time of sample collection. Therefore, it can be inferred that sample storage did not contribute to the observed variation in TNF-α expression

RECOMMENDATION

The authors in this study recommend further investigation into the effect of tissue storage on gene expression studies. Their study found no significant difference in quantified nucleic acid between RNALater stored tissue and fresh tissue. However, the authors observed a significant variation in quantitative gene expression studies. To confirm if tissue storage affects the expression of a gene in gene expression studies, the authors suggest conducting further studies using several housekeeping genes.

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