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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANALYSIS OF HOUSEKEEPING GENE STABILIZATION IN ISOLATED MITOCHONDRIA

İZOLE MİTOKONDRİLERDE HOUSEKEEPING GEN STABİLİZASYONUNUN ANALİZİ

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ABSTRACT

Objective: *The mitochondria isolation method is used to investigate mitochondrial processes. RT-qPCR measurement of gene transcription levels is frequently used in these studies. In this method, it is important that the housekeeping genes used in the normalisation of the results are stable. The study aimed to determine which of two housekeeping genes is more stable in isolated mitochondria.*
Material and Method: *Mitochondria were isolated from FHC cells. Membrane integrity and functionality were measured by mitotracker and JC-1 stainings and ROS ratio by flow cytometry. Housekeeping genes GAPDH and β-actin were used in RT-qPCR, and gene stability was calculated using ΔCq method and percentage coefficient of variance. For gene validation, CAT and SOD1 transcriptions were calculated by the $2^{-\Delta\Delta}$Cq method. These genes were selected because oxidative stress status has been detected.*
Result and Discussion: *Isolated mitochondria membrane integrity was preserved and the membrane potential ratio was found 91%. ROS ratio was 1.6% and there was no oxidative stress that would affect the antioxidant enzymes levels. ΔCq values were 4.54±0.06 for β-actin and 0.69±0.16 for GAPDH, with 1.33% and 23.95% percentage coefficient of variance respectively. CAT transcription levels were 25.7±2.6 and 1.8±0.33, while SOD1 levels were 70.1±12.7 and 4.8±0.6 correspondingly. It was concluded that β-actin may be more stable than GAPDH. The potential impact of housekeeping gene selection on outcomes should be considered.*
Keywords: *Housekeeping gene stabilization, isolated mitochondria, RT-qPCR*

ÖZ

Amaç: *Mitokondri izolasyonu metodu mitokondriyal süreçlerin araştırılmasında kullanılır. Bu araştırmalarda gen transkripsiyon seviyelerinin RT-qPCR ölçümü sıklıkla kullanılmaktadır. Bu yöntemde sonuçların normalizasyonunda kullanılan housekeeping genlerin stabil olması önemlidir. Bu çalışma, izole mitokondride iki housekeeping genden hangisinin daha stabil olduğunu belirlemeyi amaçlamıştır.*
Gereç ve Yöntem: *Mitokondriler FHC hücrelerinden izole edilmiştir. İzole mitokondrilerin membran bütünlüğü ve fonksiyonelliği mitotracker ve JC-1 boyamaları ile ROS oranı flow*

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sitometriyle ölçülmüştür. RT-qPCR analizinde housekeeping gen olarak GAPDH ve β-aktin kullanılmış, gen stabilitesi ise ΔCq metodu ve yüzde varyans katsayısına göre hesaplanmıştır. Gen validasyonu için CAT ve SOD1 transkripsiyonları $2^{-\Delta\Delta}$Cq yöntemiyle hesaplanmıştır. Bu genler oksidatif stres durumunun tespit edilmiş olması nedeniyle seçilmiştir.

Sonuç ve Tartışma: *İzole mitokondrilerin membran bütünlüğünü koruduğu görülmüş, membran potansiyeli oranı ise %91 olarak bulunmuştur. ROS oranı %1.6 olarak ölçülmüştür ve buna göre antioksidan enzim düzeyini etkileyecek bir oksidatif stres olmadığı değerlendirilmiştir. ΔCq değerleri β-aktin için 4.54±0.06 ve GAPDH için 0.69±0.16 iken, yüzde varyans katsayısı sırasıyla %1,33 ve %23.95 idi. CAT transkripsiyon seviyesi, β-actin'e göre 25.7±2.6 ve GAPDH'a göre 1.8±0.33 iken, SOD1 seviyeleri β-aktin'e göre 70.1±12.7 ve GAPDH'a göre 4.8±0.6 olarak hesaplanmıştır. Sonuçlara göre β-aktin'in GAPDH'a göre daha stabil olabileceği kanısına varılmıştır. Analizlerde housekeeping gen seçiminin sonuçlar üzerindeki potansiyel etkisi dikkate alınmalıdır.*

Anahtar Kelimeler: *Housekeeping gen stabilitesi, izole mitokondri, RT-qPCR*

INTRODUCTION

Mitochondrial isolation is a widely used method in scientific research [1]. It is an important method for investigating the role of mitochondria in physiological and pathophysiological conditions. In addition, mitochondrial transplantation (MT), which uses isolated mitochondria as a therapeutic agent, is a method that has attracted much attention in recent years and has been the subject of many studies in this context.

Although mitochondria are known as the energy source of the cell, their properties are not limited to this. It is an important player in several complex processes such as maintaining the ionic balance of the cell, synaptic transmission, immune system functions, hem metabolism, intercellular communication, and cell death [2]. Mitochondria are known to be transferred between cells in the body during physiological processes. Several features, such as a double-layered membrane allow it to be transferred [3]. It is also possible to transfer these mitochondria from outside the body. This method, called MT, has been shown to help treat important diseases such as toxic organ damage, ischemic heart damage, and neurodegenerative diseases [4].

Both the better understanding of mitochondrial processes and the increasing research on the MT method have led to a greater interest in isolated mitochondria. Mitochondria are organelles that have their own genetic material (mtDNA) and in this sense are partially independent of the nucleus [5]. Even if not encoded by mtDNA, proteins associated with mitochondrial functions can be found in mitochondria, and their mRNAs have been reported to localize together with mitochondria [6,7].

Gene expression analysis is an important method used in many areas of biological research. Polymerase chain reaction (PCR) is one of the commonly used methods in these analyses, allowing gene sequences to be duplicated and quantified. It helps to make decisions about biological processes by understanding the genes that are expressed and how they interact [8].

In PCR analysis, the transcript level of the gene under investigation is calculated using the internal housekeeping gene, which is always expressed at a consistent level. Housekeeping genes are defined as the basic group of genes necessary for the organism to survive. Another important feature is that these are genes that are consistently expressed in all cells. The stability of this gene expression is usually tested in a small number of cells and conditions. However, these tests are not yet sufficient for a large number of possibilities used in many different types of research [9]. Therefore, it is necessary to show which housekeeping gene is best suited for normalization in PCR analyses under different conditions. The fact that this gene is not affected by the interventions to be applied in the research will also allow the results to be calculated in the most accurate way.

On this basis, RT-qPCR was performed on total RNA from isolated mitochondria to determine which of two different housekeeping genes is more stable. In this study, GAPDH and β-actin were selected as housekeeping genes. These genes are among the most widely used reference genes. CAT and SOD1 enzymes were selected as target genes. These target genes were used to evaluate the effect of the calculations on the results by normalizing the transcription levels according to different housekeeping genes. The calculation of housekeeping gene stabilisation is contingent upon the selection

of a target gene for inclusion in the calculation. In the present study, the selection of these genes was facilitated by the identification of the oxidative stress status of isolated mitochondria by reactive oxygen species (ROS) analysis. ROS was measured to demonstrate that there was no oxidative stress in healthy cells and that these enzymes were at physiological levels. The objective of this research is to highlight the necessity of identifying the most suitable reference gene for use with isolated mitochondria.

MATERIAL AND METHOD

Cell Culture

Fetal human colon epithelial (FHC) cells, used as a source of mitochondria, were grown in line with basic cell culture principles. Briefly, 1×10^6 cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) with 10% fetal bovine serum, 1% l-glutamine and 1% penicillin-streptomycin in T75 culture flasks at 37°C and 5% CO_2. The medium was changed every 2 days until 70-80% confluence was reached.

Mitochondria Isolation

Mitochondria were isolated using a commercially available kit (Mitochondria Isolation Kit, ScienCell) according to the kit protocol. Approximately 2×10^7 cells were used for isolation. The isolated mitochondria were resuspended in phosphate buffered saline (PBS).

Membrane Integrity Analysis of Isolated Mitochondria

Isolated mitochondria were stained with Mitotracker (MitoSpy Green FM, Biolegend). Staining was performed by incubating 100 µl of isolated mitochondria with 200 nM mitotracker in a preheated oven at 37°C for 10 min. Then, 10 µl of stained isolated mitochondria were spread on slides and immediately visualized with a fluorescence microscope (Nikon i80, Japan) to show mitochondrial mass. The images were acquired through the utilization of a FITC filter, with a magnification of x1000 [10,11].

Flow Cytometry Analysis of Isolated Mitochondria

Flow cytometry analyses were performed using the BD Accuri™ C6 Plus. Samples were read at medium flow rate using SSC and FSC modes and 50.000 events were collected per sample. Unstained isolated mitochondria were used as a negative control. In addition, vehicle buffer was used to separate debris from mitochondria to detect the mitochondrial population. These data were used to obtain gates from the mitochondrial population. Analyses were performed after obtaining gates from individual mitochondria using the FSC vs. FSC-W graph. Subsequently, stained mitochondrial samples were analysed according to their dye properties as described below.

Mitochondrial Membrane Potential Measurement: After 100 µl of isolated mitochondria were added to 900 µl of PBS, the membrane potential was measured according to the kit protocol using a commercial kit (mitochondrial membrane potential (MMP) assay kit, Thorvacs). Samples were read at 488 nm excitation and 535 nm emission.

ROS Measurement: After 100 µl of isolated mitochondria in 900 µl PBS, ROS measurement was performed using a kit (ROS Analysis Kit, Thorvacs) according to the kit protocol. Samples were read at 485 nm excitation and 535 nm emission in the FL1 channel.

Reverse Transcription-Quantitative PCR (RT-qPCR) Analysis

The isolation of RNA from isolated mitochondria was conducted using a total RNA isolation kit (Total RNA Kit, Eco-Tech). The polymerase chain reaction was conducted using the SYBR Green dye detection method (CYBRFast™ qPCR Lo-ROX Master Mix, TONBO Bioscience) and an ABI 7500 PCR instrument (Applied Biosystem) under the default conditions. The temperature was maintained at 95°C for three minutes, followed by 40 cycles of 95°C for five seconds and 60°C for 30 seconds.

The FHC cells were employed as a control in calculation. As the genes under investigation are encoded by nuclear DNA, these cells were selected as the control for the calculation. The total mRNA was isolated from the mitochondria of these cells by the same method.

The ΔCq method was employed for the calculation of housekeeping gene expression stability [12-

14]. In this approach, the variance of a gene across all samples is calculated collectively. A constant ΔCq value between two genes indicates stable expression, whereas fluctuations indicate variable expression. In other words, the gene with the least variation between ΔCq measurements is considered to be the most stable, and this method can be used to select a suitable housekeeping gene. Furthermore, the coefficient of variance (CV) was calculated for these values. The gene exhibiting the lowest values was thus deemed to be the most stable [15].

Gene expression levels were analyzed using the $2^{-\Delta\Delta}$Cq method. GAPDH and β-actin were employed as housekeeping genes in the analysis. These genes are among the most widely utilized housekeeping genes in the domain of scientific research. They are also among the most frequently employed genes for normalization purposes in studies involving isolated mitochondria. For instance, these are among the most commonly used genes in mitochondrial transplantation studies using viable mitochondria, which is one of the most popular research areas of recent years [16-19]. However, a comprehensive study to determine housekeeping gene stabilization in mitochondrial transplantation research has not been found in the literature. Consequently, the selection of these genes was based on their relevance to ongoing research. In addition to measuring the expression levels of GAPDH and β-actin mRNA, the expression levels of CAT and SOD1, which are target genes, were also measured in both FHC cells and isolated mitochondria. The expression levels of CAT and SOD1 were calculated separately based on both housekeeping genes. In the calculation, the target genes were normalized to their own expression levels. The lowest $2^{-\Delta\Delta}$Cq value was selected as the basis for normalization.

The primer sequences employed in the RT-qPCR analysis are presented in Table 1. The primer designs were created using the Primer-BLAST® (Basic Local Alignment Search Tool) sequence matching analysis tool on the National Center for Biotechnology Information Gene Bank (NCBI-Gene Bank) database. The designs were then evaluated and refined with the Primer3 (https://primer3.ut.ee/) software.

Tablo 1. Table of primer sequences

Oligo Name	Base Sequence: 5′-3′	Reference ID
GAPDH	F: TTTTGCGTCGCCAGCC R: ATGGAATTTGCCATGGGTGGA	NCBI Reference Sequence: NM_002046.7 (208 BP)
β-actin	F: CTTCGCGGGCGACGAT R: CCACATAGGAATCCTTCTGACC	NCBI Reference Sequence: NM_001101.5 (104)
SOD1	F: AAAGATGGTGTGGCCGATGT R: CAAGCCAAACGACTTCCAGC	NCBI Reference Sequence: NM_000454.5 (167 BP)
CAT	F: ACTTCTGGAGCCTACGTCCT R: AAAGTCTCGCCGCATCTTCA	NCBI Reference Sequence: NM_001752.4 (210 BP)

RESULT AND DISCUSSION

Fluorescence microscopy images of isolated mitochondria labeled with Mitotracker demonstrate that the mitochondria retain their membrane integrity (Figure 1). Flow cytometry analysis of the membrane potential of isolated mitochondria demonstrated that 91% of the mitochondria retained their membrane potential (Figure 2). Accordingly, it is concluded that isolated mitochondria are viable. The ratio of ROS in isolated mitochondria, as determined by flow cytometry, was 1.6% (Figure 3). ROS measurement indicated a low level of ROS production and no evidence of oxidative stress.

RT-qPCR is one of the most widely used and accepted methods for quantitative gene expression analysis. The potential problems associated with the analysis of gene transcription changes can be mitigated using appropriate reference genes for normalization. One of the most used of these genes is GAPDH. However, its stability is susceptible to changes due to variations in tissue type and cellular metabolism [20]. In one study, GAPDH was identified as the housekeeping gene with the highest stability. The study assessed the stability of common housekeeping genes in porcine alveolar macrophages following stimulation with lipopolysaccharide and lipoteichoic acid. Different algorithms were used to assess the stability of these genes. The combination of SDHA, YWHAZ, and RPL4 was

identified as a potentially optimal housekeeping gene combination for precise normalization of gene expression levels in porcine alveolar macrophages [20]. However, another study investigated the IGF family gene expression at different stages of pregnancy in the feline uterus and identified GAPDH as the most stable housekeeping gene within the 8-gene design [20]. Similarly, stability analyses for β-actin and other housekeeping genes have been performed in studies using different research models [21].

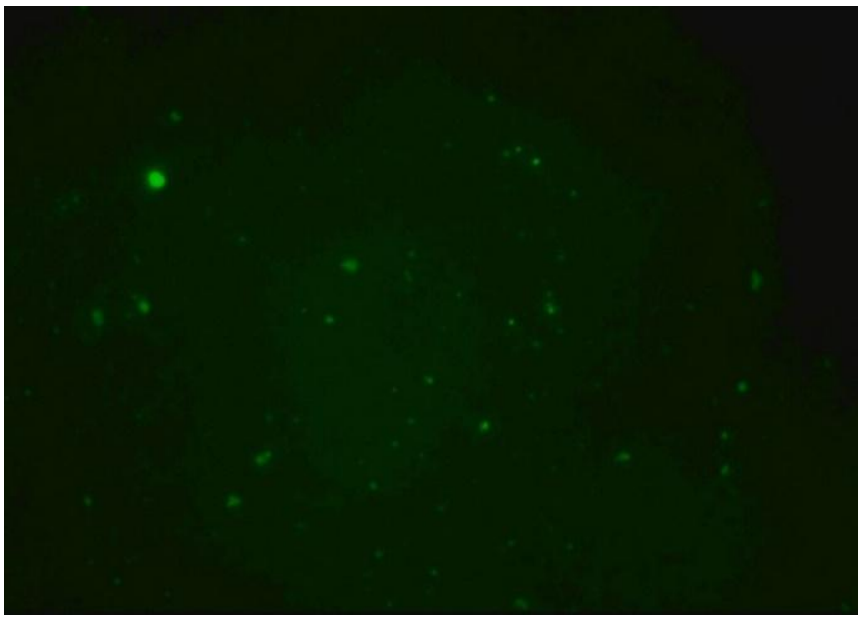

Figure 1. Fluorescence microscope image of isolated mitochondria

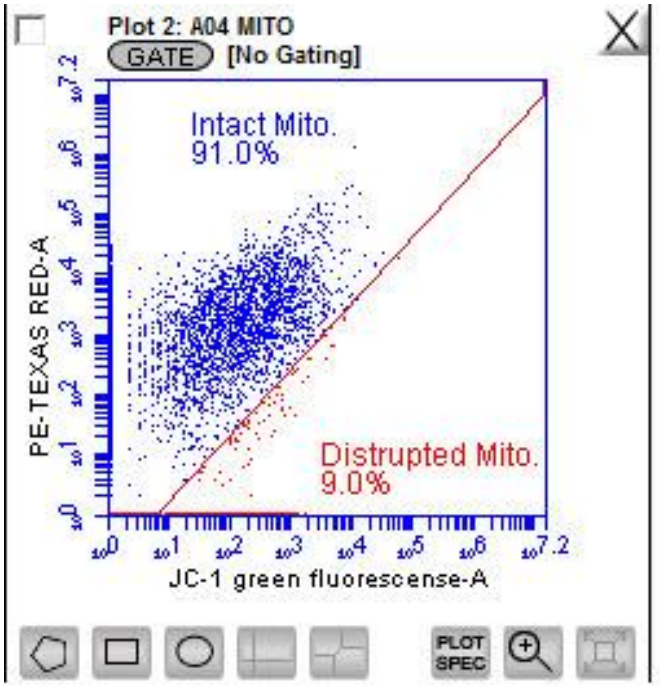

Figure 2. Flow cytometry analysis of isolated mitochondria membrane potential

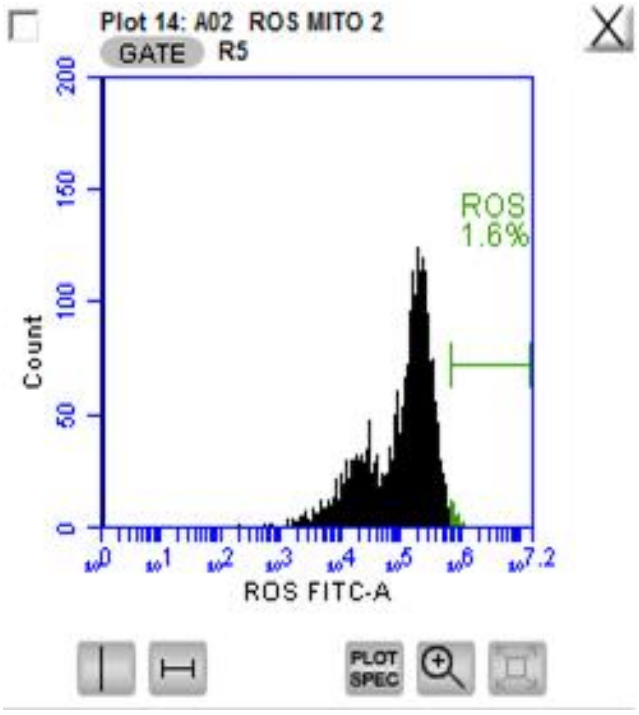

Figure 3. Flow cytometry analysis of isolated mitochondria ROS level

The objective of this study was to ascertain which of the two most commonly used genes is more stable. According to the ΔCq method used for housekeeping gene stabilization, the mean ΔCq value of β-actin was determined to be 4.54, with a standard deviation of 0.06. The mean value for GAPDH was 0.69, with a standard deviation of 0.16. The coefficient of variation (CV) was calculated to be 1.33% for β-actin and 23.95% for GAPDH (Table 2). The results demonstrated lower values in β-actin. It was therefore concluded that β-actin may be a more stable option than GAPDH among the housekeeping genes that could be used for normalization in PCR analyses.

Tablo 2. The data presented herewith pertains to the stability calculations of the housekeeping genes

	β-actin	**GAPDH**
ΔCq values	4.55	0.88
	4.48	0.61
	4.6	0.58
Standard Deviation	0.06	0.16
Mean	4.54	0.69
Coefficient of Variance (CV)	0.013	0.239
CV%	1.33	23.95

Subsequently, the transcription levels of CAT and SOD1 enzymes were calculated separately according to both housekeeping genes for validation. According to the $2^{-\Delta\Delta}$Cq method, the CAT transcription level of isolated mitochondria was calculated as 25.7 ± 2.6 according to housekeeping gene β-actin, and 1.8 ± 0.33 according to GAPDH. SOD1 transcription levels were 70.1 ± 12.7 according to β-actin, and 4.8 ± 0.6 according to GAPDH (Figure 4). Gene transcription results are presented as the mean ± standard deviation. The results indicated a 14.3-fold difference in CAT levels and a 14.5-fold difference in SOD1 levels according to the selected housekeeping gene. The findings were evaluated as calculations based on different housekeeping genes could potentially influence the results. Consequently, it reiterates the importance of selecting appropriate housekeeping genes.

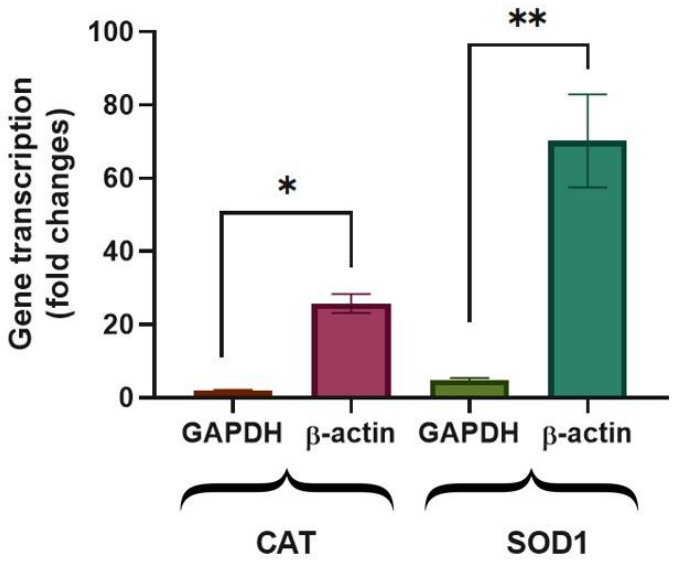

Figure 4. CAT and SOD1 enzyme transcription levels of isolated mitochondria according to different housekeeping genes. Calculations were made according to the $2^{-\Delta\Delta}$Cq method. (*) significant difference between GAPDH and β-actin in CAT calculation ($p<0.05$), (**) significant difference between GAPDH and β-actin in SOD1 calculation ($p<0.05$). Student's t-test was used in statistical analysis

It should be noted that there may be other genes that are more stable than the two housekeeping genes that were analyzed in this study. Given that mitochondria are the primary metabolic actors within cells and can influence the stability of enzymes such as GAPDH in a range of mitochondrial processes [22]. Similarly, mitochondria are highly dynamic organelles. They are closely related to other organelles and various cellular structures, both in terms of their physical and functional relationships. In general, cytoplasmic actin has been demonstrated to regulate cell morphology and dynamics, as well as exerting an influence on mitochondrial function. β-actin is essential for mitochondrial quality control, mtDNA transcription and the maintenance of mitochondrial membrane potential [23]. It can be hypothesized that these characteristics may act as factors affecting gene stability depending on the period of the cell. No comprehensive study on housekeeping gene selection in isolated mitochondria has been found in the literature. However, various studies have presented reports on the expression levels of mitochondrial genes, and in some of these studies, housekeeping gene stabilization has been determined. For instance, in a study on *Cryptolestes ferrugineus*, where mitochondrial gene expression levels were investigated, it was found that RPS13, EF1α, and γ-TUB were among the most stable genes, while α-TUB, CycA and GAPDH were among the least stable genes [24]. In another study, it was reported that ACTB, CyC1, YWHAZ and SDHA were among the stable genes for isolated and cultured limbal cells, and ATP5B, CyC1, EIF4A2, RPL13A, TOP1 and UBC were among the stable genes for isolated and cultured conjunctival cells. In this study, however, GAPDH and 18s rRNA were not identified as stable genes [25]. Mitochondrial transplantation is one of the most widely studied methods in mitochondrial research in recent years. In these studies, GAPDH and B-actin are among the most frequently used genes for normalization [16-19]. Furthermore, various genes such as 18s RNA, RPL and RNU6B have been utilized [26-28]. However, a comprehensive definition of housekeeping gene stabilization remains to be reported in these studies. Therefore, further comprehensive studies using a larger number of samples and housekeeping genes will help to select the most stable gene for mitochondrial research.

Conclusion

Mitochondria isolation is a method used in mitochondria research, which has emerged as a topic of interest in recent years. The currently defined protocols facilitate the efficient isolation of

mitochondria. These mitochondria can be employed for transplantation and subjected to analyses, including protein and ATP measurements, oxygen consumption rates, and complex analyses, with the objective of enhancing comprehension of mitochondrial processes. Moreover, gene transcription analyses of a range of mitochondria-related proteins can be conducted using isolated mitochondria. In the PCR method, which is frequently employed in gene transcription analyses, the selection of the most appropriate housekeeping gene during the analysis of the data represents a crucial step in the method. It is important to note that the selection of a housekeeping gene can potentially lead to erroneous results. Therefore, it is essential to perform analyses using the most appropriate gene. Despite the current limitations in our understanding of this subject, it is evident that further research is required on the selection of housekeeping genes in PCR analyses, particularly in the context of mitochondrial research.

AUTHOR CONTRIBUTIONS

Concept: O.U.; Design: O.U., T.F.; Control: O.U., T.F., S.O., P.M.E.; Sources: O.U., T.F., S.O., P.M.E.; Materials: O.U., T.F., S.O., P.M.E.; Data Collection and Processing: O.U., T.F., S.O., P.M.E.; Analysis and Interpretation: O.U., T.F., S.O., P.M.E.; Literature Review: O.U.; Manuscript Writing: O.U.; Critical Review: O.U., T.F., S.O., P.M.E.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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