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Effect of Mitochondrial Transplantation on Reference Gene Stability in Healthy FHC Cells

Sağlıklı FHC Hücrelerinde Mitokondri Transplantasyonunun Referans Gen Stabilizasyonu Üzerindeki Etkisi

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ABSTRACT

Objectives: RT-qPCR is frequently used to determine gene expression levels. Expression levels are calculated relative to consistently expressed reference genes. Although Gapdh and Actb are commonly used reference genes in mitochondrial transplantation (MTx) studies, no comprehensive research on gene selection has been found.

Materials and Methods: Healthy Fetal Human Colon (FHC) cells were used for MTx and mitochondrial isolation. Quality control was performed by measuring protein, ATP, membrane potential, and ROS levels in isolated mitochondria. Gapdh, Actb, Sod, and Cat levels were measured after MTx to evaluate and validate gene stability. The CV and ΔCq methods were used in stability analyses, along with the BestKeeper tool.

Results: Isolated mitochondrial protein, ATP, MMP, and ROS levels were measured as 52.23 µg/mL, 533.58 nmol, 92%, and ROS 2%, respectively. In CV calculations, Gapdh was found to be more consistent than Actb (2.15% versus 2.72%). The ΔCq method showed Actb to be slightly more stable (0.45 versus 0.46). The Bestkeeper found that the variation was lower for Gapdh (1.86% vs. 2.20%), but Actb was more consistent with the Bestkeeper index (0.982 vs. 0.971). Calculating the expression levels of Sod and Cat for different genes led to statistically significant changes.

Conclusion: According to the results, both genes are stable. However, Gapdh shows less variability, while Actb is more correlated with the BestKeeper index. GAPDH is a proper choice due to its low variation, but a combination of genes may provide more reliable normalization. The research findings indicate that the choice of reference gene can affect the results and that the stability of the reference gene must be checked to obtain reliable results.

Keywords: mitochondrial transplantation, reference gene, gene stabilization

ÖZET

Amaç: Gen ekspresyon seviyelerinin belirlenmesinde RT-qPCR sıklıkla kullanılmaktadır. Ekspresyon seviyeleri tutarlı bir şekilde eksprese edilen referans genlere göre hesaplanır. Mitokondri transplantasyonu (MTx) çalışmalarında Gapdh ve Actb sık kullanılan genlerden olsa da gen seçimi konusunda kapsamlı araştırmaya rastlanılamamıştır.

Gereç ve Yöntem: MTx ve mitokondri izolasyonu için sağlıklı Fetal İnsan Kolon (FHC) hücreleri kullanılmıştır. İzole mitokondrilerin protein, ATP, membran potansiyeli ve ROS ölçümleri ile kalite kontrolü yapılmıştır. MTx sonrası gen stabilitesini değerlendirmek ve doğrulamak için Gapdh, Actb, Sod ve Cat seviyeleri ölçülmüştür. Stabilite analizlerinde CV ve ΔCq yöntemlerinin yanında BestKeeper aracı kullanılmıştır.

Bulgular: İzole mitokondri protein, ATP, MMP ve ROS düzeyleri sırasıyla 52.23 µg/mL, 533.58 nmol, %92 ve ROS %2 olarak ölçülmüştür. CV hesaplamasında Gapdh'nin Actb'den daha tutarlı olduğu görülmüştür (%2.15'e karşı %2.72). ΔCq yöntemi, Actb'nin (0.45'e karşı 0.46) biraz daha stabil olduğunu göstermiştir. Bestkeeper, Gapdh için varyasyonun daha az olduğunu (1.86%'ya karşı 2.20%), ancak Actb'nin Bestkeeper indeksine daha uyumlu (0.982'ye karşı 0.971) olduğunu göstermiştir. Farklı genlere Sod ve Cat ekspresyon seviyelerinin hesaplanmasının istatistiksel olarak anlamlı değişimlere yol açmıştır.

Sonuç: Sonuçlara göre her iki gen de stabildir. Ancak Gapdh daha az değişkenlik gösterirken, Actb BestKeeper indeksi ile daha koreledir. GAPDH, düşük varyasyonu nedeniyle uygun bir seçimdir, ancak genlerin kombinasyonu daha güvenilir normalizasyon sağlayabilir. Araştırma bulguları referans gen seçiminin sonuçları etkileyebileceğini ve güvenilir sonuçlar alınması için referans gen stabilitesinin kontrol edilmelisi gerektiğini göstermektedir.

Anahtar Kelimeler: mitokondri transplantasyonu, referans gen, gen stabilitesi

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Introduction

Reverse transcription-quantitative PCR (RT-qPCR) is regarded as a gold standard in biological research for the examination of gene expression levels. The method involves the detection of RNA and subsequent quantification. For this purpose, complementary DNA (cDNA) produced from RNA extracted from the sample is utilized as a template, and the target gene is amplified. RT-qPCR is a highly sensitive and effective method that is used widely. This method has been employed for a variety of purposes, including gene expression studies, pathogen recognition, genetic analysis, and disease investigation. The expression level of a gene is calculated by normalizing it to a reference gene. This methodology facilitates the calculation of expression levels in relation to a specific gene, whilst concomitantly reducing measurement errors that may be attributable to variations in sample quality and RNA extraction methodologies. Consequently, the utilization of a stable reference gene exhibiting relatively constant expression across diverse tissues and cells assumes a pivotal role in target gene analysis (1).

Reference genes are commonly chosen from among housekeeping genes that are responsible for essential cellular activities such as cell cycle regulation, DNA replication, and metabolism. It is noteworthy that these are invariably expressed at a continuous level within a cell (2). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Actin beta (Actb), 18S RNA (18S), 28S rRNA (28S), tubulin, hypoxanthine–guanine phosphoribosyl transferase (Hprtp), and beta-2-microglobulin (B2m), elongation factor 1-alpha (Ef1-α), and ubiquitin conjugating enzyme (UbcE2) are some of the reference genes commonly used for this purpose (3, 4).

Despite the prevailing consensus that housekeeping genes are generally stable across different tissues and conditions, there is considerable variation in their transcription levels across species, treatments, and/or developmental stages. Indeed, experiments have found that the expression level of these could vary depending on stress factors such as cell type, organism type, experimental conditions, and drugs administered (5). This means that a single reference gene cannot be used universally. It is therefore imperative to establish the stability of these normalization genes employed in PCR analysis, thereby ensuring the accurate determination of target gene expression. As the selection of reference genes directly impacts the calculated target gene expression level, it is imperative to verify the expression stability of reference genes under the experimental conditions employed (3). An ideal reference gene should be constantly expressed and not affected by experimental conditions (6).

Various gene stability calculation methods, such as delta Ct (7), coefficients of variation (CV) (8) and software tools such as geNorm (9), NormFinder (10), BestKeeper (11) and RefFinder (12) are available (13). These approaches facilitate the identification of genes that exhibit greater stability in their expression levels through a series of calculations. The delta Ct method is predicated on the evaluation of the comparative expressions of reference genes. A constant delta Ct value between two genes would suggest that those genes' expression is steady (7). In the CV method, the variability of reference genes is determined by coefficient of variation, determined by dividing the standard deviation by the mean. This method provides a variability calculation that is not affected by the average expression level of the gene. A low CV value is indicative of higher gene stability (8). The GeNorm program has been developed to determine gene stability. This is achieved by calculating an M value using raw Cq values. The average pairwise variance between genes is known as the M value. A lower M value means better stability in this method (9). NormFinder is a Microsoft Excel tool that performs a stability calculation based on a combined estimate of intra-/inter-group variance (10). BestKeeper is a Microsoft Excel spreadsheet instrument that evaluates stability by calculating various statistical data, including arithmetic mean (AM), geometric mean (GM), standard deviation (SD), correlation coefficient (r), and coefficient of variation (CV) from raw Cq values. In this method, reference gene selection is predicated on the identification of a gene that exhibits a lower standard deviation (SD) and a higher r value (11).

Mitochondrial transplantation (MTx) has emerged as a highly promising field in recent years. Efforts to understand of the critical roles of mitochondria in maintaining cellular homeostasis and their role in the development of many diseases has gained momentum over the past half-century (14, 15). The discovery that mitochondria are transferable organelles between cells and the subsequent understanding of their role in various biological processes have led to the consideration of artificially performing this process externally. In recent years, it has been observed that this process, which can be referred to as MTx, has the capacity to affect crucial cellular repair mechanisms. The beneficial effects of this process have been demonstrated to include reduced cell death, increased regeneration, and the prevention of oxidative stress and inflammation (16). These results have led to a noteworthy upsurge in the pace of research.

PCR analysis is a common method used to investigate the mechanism of action of MTx. In the present studies, *Gapdh*, *Actb*, and *18S* are frequently utilized as reference genes for normalization (17-20). Yet, a complete examination of the reference gene selection and gene stability specific to MTx has not yet been conducted. In the present study, the impact of exogenous mitochondria transplanted into healthy human colon epithelial cell lines (FHC) on the stability of the *Gapdh* and *Actb* genes, which are the most utilized reference genes, was examined.

Materials and methods

Cell culture

The FHC cell line (ATCC- CRL-1831 ™) was used in this study. Cells were cultured in T75 culture flasks in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% l-glutamine and 1% penicillin-streptomycin at 37°C and 5% CO2 in an incubator (21, 22). The cells were divided into three groups: Control, MTx (1 µg), and MTx (10 µg). Three biological replicates were used in the study, and analyses were performed using three technical replicates.

Mitochondria isolation

Mitochondria isolation was performed using Fetal Human Colon (FHC) cells and a commercial mitochondria isolation kit (Abcam, ab288084). Briefly, cells (2×10^7 cells) were collected and washed, then 1 ml of "Mitochondria Isolation Buffer" was added and incubated on ice for 2 minutes. Then, 10 *µl* of "Permeabilization Reagent" was added and incubated on ice for 5 minutes. The pellet was then centrifuged at 600 x g for 10 minutes at +4 °C, and the supernatants were separated and centrifuged at 7,000 x g for 10 minutes at +4 °C. After removing the supernatant, the mitochondrial pellet was washed using "Mitochondria Isolation Buffer" and suspended in "Storage Buffer" for use.

Mitochondrial transplantation

Isolated mitochondria were transplanted into FHC cells via simple co-incubation. Approximately 1 *µg* and 10 *µg* of mitochondria were transplanted into $1x10^6$ FHC cells in 4 mL of medium in each plate. To the best of our knowledge, as there are no studies on MTx in FHC cells, the mitochondrial doses used in our study were determined based on previous studies. In an in vitro study, mitochondrial doses of 0.05, 0.5, and 5 *µg* mitochondria / 1×10^5 recipient cells were utilized in L6 cell line (23). In a further study, mitochondria obtained from UC-MSCs were transplanted into tenocyte cells at doses of 1, 5, and 25 *µg* / 1×10^5 cells (24). Accordingly, the doses utilised in the present study were established at 1 and 10 *µg*, in consideration of the fact that the cells originated from a healthy epithelial cell line. The experiment was terminated at 24 hours post-transplantation, and the cells were collected for analysis. In the context of in vitro studies, a 24-hour period is frequently employed following MTx. A study on brain organoids reported that transplanted isolated mitochondria entered cells within two hours, and mtDNA levels were analyzed 24 hours after MTx (25). Furthermore, a study on chondrocytes, a healthy fibroblast cell line, performed flow cytometry, protein, and gene transcription analyses after 24 hours waiting period after MTx (26).

Isolated mitochondria characterization

Quality control characterization was achieved to determine the suitability of isolated mitochondria for MTx and doses. For this purpose, mitochondria isolated from $5x10^6$ cells were suspended in 200 *µl* of buffer.

Protein measurement: Protein concentration of the mitochondria was determined with the BCA Pro-

Table 1: Gene Primers

Primers	Reference	Sequences
Gapdh	NCBI Reference Sequence: NM_002046.7 (208 BP)	**F:** TTTTGCGTCGCCAGCC **R:** ATGGAATTTGCCATGGGTGGA
Actb	NCBI Reference Sequence: NM_001101.5 (1812)	**F:** ACACAGTGCTGTCTGGTGGT **R:** TGATCTTCATGGTGCTGGGAG
Sod1	NCBI Reference Sequence: NM_000454.5 (167 BP)	**F:** AAAGATGGTGTGGCCGATGT **R:** CAAGCCAAACGACTTCCAGC
Cat	NCBI Reference Sequence: NM_001752.4 (210 BP)	**F:** ACTTCTGGAGCCTACGTCCT **R:** AAAGTCTCGCCGCATCTTCA

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; Actb: Actin beta; Sod1: Superoxide dismutase 1; Cat: Catalase.

tein Assay Kit (Thermo Scientific™, A65453) according to the kit protocol. Measurements were performed on samples containing 25 *µl* of mitochondria. Readings were taken at a wavelength of 562 nm using a microplate reader (Synergy H1, BioTek Instruments).

ATP measurement: ATP concentration was measured with the ATP Detection Assay Kit (Cayman, 700410). The measurements were performed using 10 *µl* of mitochondrial sample, and the luminescence value was measured using a microplate reader.

Membrane potential measurement: 50 *µl* of mitochondrial samples added to 500 *µl* of buffer were analyzed using the mitochondrial membrane potential (MMP) assay kit (Thorvacs, MMPAK-100T). 5 *µl* of JC-1 stock solution was incubated with samples in the dark at 37 °C for 30 minutes. The samples were analyzed (FACSCalibur system, BD Biosciences) using the FL 1 channel.

Reactive oxygen species (ROS): ROS measurements of mitochondria were performed using the ROS Analysis Commercial Kit (Thorvacs, ROS-110T). 50 *µl* of mitochondria in 500 *µl* of buffer is used for analysis. Readings were taken using a flow cytometer using the FL 1 channel.

RT-qPCR analysis

Total RNA isolation from FHC cells was performed with the Total RNA Kit (Eco-Tech, E2075) for PCR analysis. RNA samples which have OD260/OD280 ratio between 1.9 and 2.1 were included in the analyses. cDNA synthesis was performed from these samples using the commercial iScript™ cDNA Synthesis Kit (Biorad, 1708891), and after characterization of the samples, all samples were adjusted to equal concentrations. Polymerase chain reaction was performed using CYBRFast™ qPCR Lo-ROX Master Mix (Tonbo, 3151010100R) on an ABI 7500 PCR instrument (Applied Biosystem). *Gapdh*, *Actb*, and *Gapdh+Actb* (geometric mean of the Cq values) were used as reference genes. Sod1 and Cat genes were used to verify gene stabilization. The primer sequences are presented in Table 1.

Determination of reference gene stability

Reference gene stability calculations were performed utilizing coefficients of variation (CV) (8), delta Cq (*ΔCq*) (7), and BestKeeper (11) methods.

CV method: CV% value was calculated based on the arithmetic mean and standard deviation of all Cq values. The gene with the lower CV% value was found to be more stable.

***ΔCq* method:** The average and SD values of the *ΔCq* values obtained by subtracting the combinations of average Cq values of reference genes in different groups were used for the calculation. More stability was attributed to the gene with the lowest SD value.

BestKeeper method: This method enables stability decisions to be made based on statistical data obtained by processing Cq values using the Excel tool. According to this method, the gene with the lowest SD and CV% values and the r value closest to 1 is considered the most stable.

Verification of reference gene stability

To assess whether the results of fold change calculations based on different reference genes caused differences, expression levels of the *Sod* and *Cat* were cal-

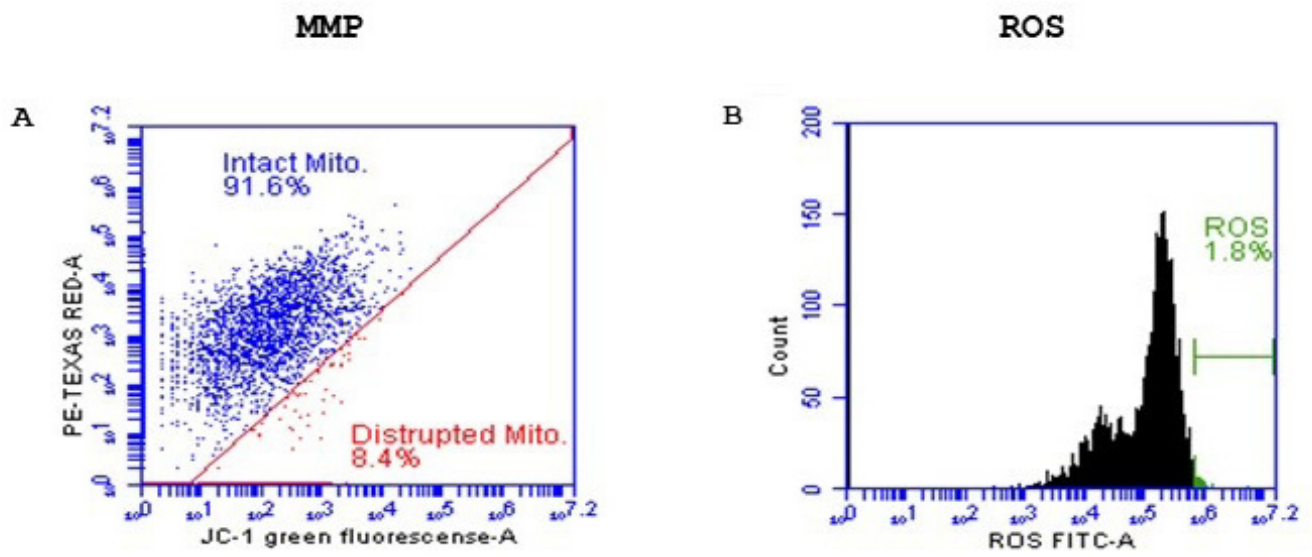


Figure 1A: Flow cytometry analysis of isolated mitochondria. MMP (A) and ROS (B) levels of isolated mitochondria. MMP: Mitochondrial membrane potential; ROS: Reactive oxygen species.

culated using $2^{-\Delta\Delta Cq}$ method. To interpret the differences between the calculations, the repeated measures ANOVA test was used as the statistical method. GraphPad Prism (version 8.0.2) was used in the analysis.

Results

Isolated mitochondria characterization results

The results of protein measurements to determine the amount of isolated mitochondria and ATP measurements to evaluate their functionality are presented in Table 2.

Table 2: Isolated mitochondria protein and ATP levels

Isolated mitochondria characterization	
Protein amount (µg/mL)	52.23
ATP concentration (nmol/10 µL mitochondria)	533.58

ATP: Adenosine triphosphate

According to the mitochondrial membrane potential (MMP) measurement results, approximately 92% of isolated mitochondria maintained their membrane potential (Figure 1A). The reactive oxygen species (ROS) ratio was found to be approximately 2% (Figure 1B).

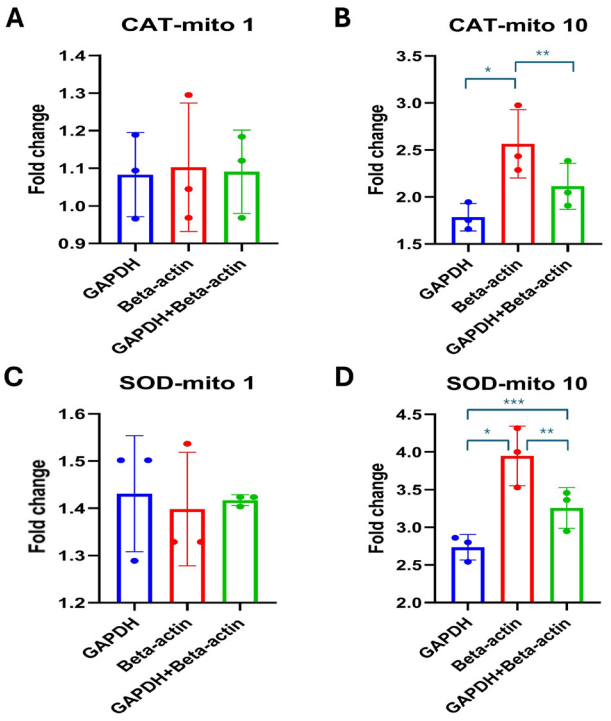


Figure 1A: Fold change levels according to different reference genes. *Cat* expression levels in the MTx (1 µg) group (A), *Cat* expression levels in the MTx (10 µg) group (B), *Sod* expression levels in the MTx (1 µg) group (C), *Sod* expression levels in the MTx (10 µg) group (D). (*) significant difference between *Gapdh* and *Actb* ($p < 0.05$), (**) significant difference between *Actb* and *Gapdh+Actb* ($p < 0.05$), (***) significant difference between *Gapdh* and *Gapdh+Actb* ($p < 0.05$). Cat: Catalase; MTx: Mitochondrial transplantation; Sod: Superoxide dismutase, Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; Actb: Actin beta.

Reference gene stability calculation findings

The CV method was primarily utilized in gene stability analyses. The data pertaining to SD and CV calculations is presented in Table 3. Consequently, the SD was determined to be 0.49 for *Gapdh* and 0.69 for *Actb*. The CV% values were calculated as 2.15% and 2.73%, respectively. It is evident from the calculations performed on all Cq values that the SD and CV% values indicate a lower level of variability in *Gapdh* expression.

Table 3: CV calculation data

	Gapdh	*Actb*
Mean	22.67	25.20
SD	0.49	0.69
CV	0.0215	0.027277
CV%	2.150028	2.727724

SD: Standard Deviation; CV: Coefficients of variation; Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; Actb: Actin beta

In the calculation performed using the *ΔCq* method, the SD values were found to be 0.46 for *Gapdh* and 0.45 for *Actb*. The calculation data are presented in Table 4. According to results both genes showed similar stability, but *Actb* showed slightly better stability.

Table 4: ΔCq calculation data

Mean Cq Values		
	Gapdh	Actb
Control	22.03	24.40
MTx (1 µg)	22.90	25.23
MTx (10 µg)	23.07	25.97
ΔCq Values		
Control- MTx (1 µg)	-0.87	-0.83
Control- MTx (10 µg)	-1.03	-1.57
MTx (1 µg)-MTx (10 µg)	-0.17	-0.73
Mean (ΔCq)	-0.69	-1.04
SD (ΔCq)	0,46	0,45

Cq: Threshold cycle; MTx: Mitochondrial transplantation; ΔCq: delta Cq; SD: Standard Deviation; Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; Actb: Actin beta

The Bestkeeper analysis results are presented in Table 5. According to this, the SD values of both reference genes are < 1, and the CV% value is < 5. This indicates that both analyzed genes are stable. However, *Gapdh* has lower SD and CV% values. The r value is > 0.9 in both genes and shows a high correlation with the BestKeeper index. In terms of correlation, *Actb* was found to have a better value than *Gapdh* and to show higher compatibility. The results indicate that both genes exhibit high stability, with *Gapdh* showing less variation, while *Actb* shows higher correlation with other genes according to the BestKeeper index.

Table 5: Bestkeeper calculation data

	Gapdh	*Actb*
geo Mean [CP]	22.66	25.19
ar Mean [CP]	22.67	25.20
min [CP]	21.90	24.30
max [CP]	23.10	26.10
std dev [± CP]	0.42	0.56
CV [% CP]	1.86	2.20
coeff. of corr. [r]	0.971	0.982
coeff. of det. [r^2]	0.943	0.964
intercept [CP]	2.807	-3.125
slope [CP]	0.831	1.185
SE [CP]	±0.124	±0.139
p-value	0.001	0.001
Power [x-fold]	1.78	2.27

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; Actb: Actin beta

Validation findings of reference gene stability

To verify the reference gene stabilization, the *Sod* and *Cat* gene transcription levels were calculated by means of the $2^{-\Delta\Delta Cq}$ method. The calculation of gene expression fold changes was performed using various reference genes and gene combinations (*Gapdh, Actb, and Gapdh+Actb*). The results are presented in Table 6.

Table 6: Fold change levels according to different reference genes

Fold Change				
		Gapdh	***Actb***	***Gapdh+Actb***
Cat	MTx (1 µg)	1.08	1.10	1.09
	MTx (10 µg)	1.79	2.57	2.11
Sod	MTx (1 µg)	1.43	1.40	1.42
	MTx (10 µg)	2.74	3.95	3.26

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; Actb: Actin beta; Sod1: Superoxide dismutase 1; Cat: Catalase; MTx: Mitochondrial transplantation.

Furthermore, the study sought to ascertain whether there existed a statistical discrepancy between calculations made according to different reference genes. The findings of the study indicate that no substantial disparities were identified between the calculations performed in the MTx (1 µg) groups. However, in the MTx (10 µg) group, significant differences were detected in *Cat* expression levels between *Gapdh* and *Actb*, and between *Actb* and *Gapdh+Actb* ($p < 0.05$). A statistically significant discrepancy was identified in the levels of *Sod* across all calculations ($p < 0.05$).

Discussion

In recent years, significant research has been conducted to understand the role of mitochondria in many physiological processes and diseases. Due to their key roles in cell physiology, many studies have been conducted on the beneficial effects of transplanting mitochondria into damaged cells/tissues from outside sources. Beneficial effects have been identified in important areas central to diseases, such as reduced cell death, increased regeneration capacity, and reduced oxidative stress. PCR analyses constitute an important analytical method for evaluating the results of these studies. In PCR analysis, the expression levels of the gene being studied are calculated relative to a reference gene. This reference gene should have characteristics such as not being affected by the experimental protocol, being stably always expressed, and being highly expressed. Since mitochondrial transplantation can directly and widely affect cell metabolism, the selection of the reference gene to be used in these studies is important. However, no comprehensive study has been found about reference gene selection in MTx experiments. In our study, we investigated whether MTx application to healthy FHC cells affects the stability of the *Gapdh* and *Actb* genes, which are among the most used reference genes.

For this purpose, mitochondria isolated from FHC cells were transplanted into healthy FHC cells at low (1 *µg*) and high (10 *µg*) doses. First, the quantity and functionality of the isolated mitochondria were analyzed (27-30). It was observed that the isolated mitochondria maintained ATP levels, preserved membrane potential, and had low ROS levels. These results indicate that mitochondrial isolation was successful and that the isolated mitochondria are suitable for transplantation. Maintaining the viability of isolated mitochondria is a critical step in MTx research. It has been reported that mitochondria or mitochondrial structures that do not maintain their viability have no positive effects on cells (31). Furthermore, it has been reported that some damaged mitochondria or mitochondrial content may act as a damage signal, triggering various harmful mechanisms (32). In our study, these viable isolated mitochondria were transferred to FHC cells using the co-incubation method.

To demonstrate how isolated mitochondria after MTx affect the expression stability of the *Gapdh* and *Actb* genes, the CV and *ΔCq* methods were used alongside the Bestkeeper calculation tool. In the CV method, Cq values from all conditions were used to calculate SD and CV% values. In PCR analyses, it is a general approach to consider the variability of all Cq values when calculating gene stability. This is because the gene should not be affected by the experimental conditions (8). The results show that *Gapdh* expression levels are less variable and more stable than *Actb*.

The *ΔCq* calculation was conducted using the PCR gene stability analysis method as delineated by Silver et al. (2006) as a fundamental framework (7). In this method, the differences between the Cq values of the candidate reference gene pairs are calculated to evaluate the consistency of a reference gene's expression levels. The reference gene with smaller *ΔCq* standard deviation values between samples is considered more stable because it shows less variation in expression. In our study, the average Cq values of

the groups were used, and the *ΔCq* values were obtained by subtracting the average Cq values of the binary combinations of the Cq values of each reference gene in different groups from each other. The SD values were calculated separately for *Gapdh* and *Actb* using the values obtained in this way. The SD values showed that both genes exhibited similar stability, but *Actb* exhibited slightly better variability.

In the BestKeeper method, statistical calculations such as mean, SD, CV%, and r values are performed on all Cq values, and the variability of each gene and its correlation with the BestKeeper index are determined (11). Using the data provided in the output file, it is possible to determine which genes are the most stable (33). According to the analysis results, the SD values of both reference genes being < 1 indicates that the genes are stable (34). A lower standard deviation (SD) value indicates greater consistency in the gene expression levels across different samples. In terms of CV%, which indicates Cq variability as a percentage, both genes are below 5% and have good variability data (10, 33). As this value is reduced, there is an increase in the stability of the gene. Although both genes are stable, *Gapdh* has lower values than *Actb* and shows less variability between samples. In terms of the correlation coefficient (r), both genes showed high correlation with the BestKeeper index (33), but *Actb* was found to show higher compatibility than *Gapdh*.

The results obtained from all these different methods indicate that both genes are stable. *Gapdh* is considered more consistent among samples because it shows less variation. *Actb*, on the other hand, is more consistent with the general gene expression dynamics because it shows a high correlation with genes according to the BestKeeper index. It may be considered a statistically strong reference in the current experimental model.

In our study, the effect of calculating *Sod* and *Cat* expression levels according to different reference genes on the results was demonstrated to validate the reference gene selection. Calculations were performed using *Gapdh* and *Actb* reference genes. In addition, calculations were also performed using the *Gapdh+Actb* combination. The use of the geometric mean of the Cq values of different genes as reference genes is a common approach (Nascimento et al., 2015). In our study, although both genes were stable, different results were obtained in terms of variation and correlation. Therefore, a calculation was performed based on the average of both genes, and it was demonstrated whether the results caused statistically significant differences.

Accordingly, it has been observed that, especially in the high-dose MTx group, the calculation of gene expression levels according to different genes leads to statistically different results. These results indicate that MTx application may affect reference gene stability. In our study, both genes were found to be stable, but *Gapdh* showed less variation. Therefore, it was observed that normalization based on multiple reference genes could yield more reliable results; however, if working with a single gene is necessary, *Gapdh* may be more suitable than *Actb*. However, further studies of larger sample sizes and under different conditions are needed.

Conclusion

As a result, it was concluded that the expression levels of both genes remained stable after MTx in healthy FHC cells and were suitable as reference genes. However, if a single reference gene is to be used and a choice must be made, *Gapdh* may be more appropriate because it has less variation. Nevertheless, it can also be considered that calculations using a combination of both genes would provide a more reliable normalization. Despite all this, studies with higher sample sizes will enable the best results to be obtained. Therefore, it is important for MTx research to work with the same experimental design and higher sample sizes, as well as to perform stability analyses in different cells and under different experimental conditions.

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