Original Article

Evaluation of the Housekeeping Genes; β-Actin, Glyceraldehyde-3-Phosphate-Dehydrogenase, and 18S rRNA for Normalization in Real-Time Polymerase Chain Reaction Analysis of Gene Expression in Human Adipose Tissue

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Abstract

Background: Several studies suggested that beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rRNA are expressed constitutively and contribute to the fundamental reference actions essential for cell viability and maintenance. However, there are inconsistency in this regard. Hence, we aimed to evaluate the accuracy of these three potential reference genes for Real-Time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) application for normalization in two types of human adipose tissues. **Materials and Methods:** Subcutaneous and visceral fat tissues were derived from 19 healthy and 20 obese subjects and RT-qPCR was applied to determine the expression levels of beta-actin, GAPDH, and18S rRNA. **Results**: The gene expression level of beta-actin, GAPDH, and 18S rRNA was essentially the same in the subcutaneous and visceral fat tissues of all participants (P>0.05). Hence, all considered housekeeping genes displayed high expression stability and the analysis revealed that normalization to all of these three housekeeping genes gave a result that satisfactorily reflected the acceptable mRNA expression levels in adipose tissues. **Conclusion:** Collectively, our findings suggest of beta-actin, GAPDH, and18S rRNA as reference genes applicable in human adipose tissue in the context of obesity.

Keywords*:* Housekeeping gene, Beta-actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR).

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Introduction

The Real-Time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) technique provides the opportunity to detect the differences of gene expression at the mRNA level in different situations. Evaluation of gene expression at the mRNA level and comparative measurements of mRNA expression level in different cells and tissues need approaches for correct normalization [1]. Since cell counting is almost an impossible way for expression analysis, such measurements have to be

combined with applicable establishments of mRNA for housekeeping genes. Hence, in order to determine the relative quantification of the studied mRNA by the RT-qPCR technique, we should analyze the expression of our target mRNAs relative to a reference gene for removing any unwanted effects or variations in mRNA levels of the specific tissue. A proper housekeeping gene has some definite features including a stable and unregulated expression in the experimented cell [2-4]. Significantly, the process of gene normalization has this condition that the expression level of the housekeeping gene does not differ significantly during cell cycle or in altered experimental situations. Consequently, before choosing a gene as a reference, a comprehensive examination is required to confirm that no significant regulation would happen. Nevertheless, it may be a problem to deal with, since the mRNA expression results of the investigated reference gene has to be standardized itself [5, 6].

Several studies suggested that beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rRNA are expressed constitutively and contribute to the fundamental reference actions essential for cell viability and maintenance. Consequently, housekeeping genes such as beta-actin, GAPDH, and 18S rRNA are often applied as internal standards for RT-qPCR techniques [7, 8]. However, requirements for their appropriate use are continual expression among different cells of one type, along with the stability of expression levels against different biological states and environmental factors including different *in vitro* treatments. Nevertheless, it should be noted that the expression levels of these genes may be different in various types of tissue and also in altered conditions acting on the entire organism. This issue is investigated by several experimental animal studies. Remarkably, it is reported that the mRNA levels of beta-actin, GAPDH, and 18S rRNA may vary in human adipose tissues in different biological and pathological conditions [8-13]. Likewise, modifications in housekeeping gene expression levels may be expected in subcutaneous and visceral adipose tissue upon metabolic and inflammatory diseases. Therefore, unawareness of these differences in the mRNA levels of reference genes applied for normalization may result in the misinterpretations of analyses in the investigation of related disorders [14]. Although there is increasing evidence for selction of these genes as internal controls in studies of metobolic disordres especially obesity-related disease, there is still a great deal of uncertainty in this regard. More importantly, the structural, functional, and anatomical differences between subcutaneous and visceral adipose tissues highlight to select appropriate housekeeping genes to asssess gene expression. Moreover, there is a limited number of studies on evaluation of aforementiond genes simultaneously in both subcutaneous and visceral adipose tissues. Henceforth, in this study, we aimed to explore whether the expression levels of beta-actin, GAPDH, and 18S rRNA are fluctuating in subcutaneous and visceral adipose tissue of healthy and obese subjects using RT-qPCR.

Methods

Study Population. The study was conducted on 39 women from who referred to Erfan, Loqman Hakim, and Sina hospitals, Tehran, Iran for bariatric surgery (Roux-en-Y gastric bypass and vertical sleeve gastrectomy) or elective cholecystectomy and inguinal hernia, all women aged between 20 to 53 years old including 20 obese subjects (BMI ≥35 kg/m^2), and 19 normal-weight subjects (BMI ≤ 25 Kg/m^2).

Table1. Forward and reverse primers used for real-time PCR..

Primer	Forward sequence	Reverse sequence	Product size	Tm
beta-actin	5'-TCCTTCCTGGGCATGGAGT-3'	5'-ACTGTGTTGGCGTACAGGTC-3'	81	83
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	87	82
18 srRNA	5'-GGAGAGGGAGCCTGAGAAAC -3'	5'- CAATTACAGGGCCTCGAAAG -3'	127	83

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

The exclusion criteria were defined as any diagnosis with renal and liver disease, type 2 diabetes, cancer, cardiovascular disease, or having an operation in last 6 months. To eliminate the probable outcome of post-menopause on the mRNA expression pattern, we excluded the post-menopausal women at the beginning of the study. This study was permitted by Ethics Committee of the Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1397.827) and prior to study, written informed consent was provided from each participant.

Adipose Tissue Samples. Subcutaneous and visceral adipose tissue were collected during the specific surgeries from the participants. In details, a specialist surgeon derived 0.5 grams of visceral and subcutaneous adipose tissues from the omentun and under the skin of participants, respectively. Next, the collected samples were washed in sterile phosphate-buffered saline, directly frozen in liquid nitrogen, and stored at -80°C until further investigations.

RT-qPCR. After homogenization of subcutaneous and visceral adipose tissue samples separately, the total RNA was extracted by RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Germany), based on the manufacturer's protocol. The determination of OD260/230 and OD260/280 ratios determined the purity of extracted RNA. Moreover, the quality and integrity of RNA were measured to show the appropriateness of the extracted RNA for RT-qPCR. Next, 1µg of total RNA was aplied as a template for first-strand complementary DNA (cDNA) synthesis by PrimeScript 1st Strand cDNA Synthesis kit (Takara, Japan) according to the manufacturer's protocol. Finally, RT-qPCR was performed in a Step-One-Plus TM Real-Time (ABI Applied Biosystems) by BioFACT™ 2X Real-Time PCR Master Mix (For SYBR Green I) using primers in Table 1 based on the manufacturer's protocol.

Standard curves were generated for all studied genes to evaluate the linear range of the real-time PCR before performing the assay on test samples. The correlation coefficients of all the standard curves were more than 0.95 (R^2) indicating a good linearity, and all test samples were verified to be within this range.

Anthropometric and Biochemical Measurements. The anthropometric indices of all participants were measured, including WC, hip, waist-to-hip-ratio (WHR), weight, height, and BMI. BMI was calculated based on the ratio of weight in kg divided by height in m^2 to assess the fatness of participants. WHR was calculated according to the ratio of WC in cm divided by hip circumference in cm.

Biochemical and Laboratory Measurements. Serum was separated from blood samples obtained from participants after an overnight fasting. The separated serum was stored frozen at -80°C until next experiments. Aspartate amino transferase (AST) and alanine amino transferase (ALT) were analyzed by auto analyzer using commercial kits (Pars Azmoon, Tehran, Iran). The fasting plasma insulin was measured by ECL method in Cobas6000 E601 auto analyzer. Any other biochemical parameters were measured by enzymatic process using assay kit adapted in hospitals.

Statistical Analysis. Results were showed as mean ± standard error of mean (SEM). The normality of data was checked by using the Shapiro-Wilk test and the Student's t-test was used for comparing the significance of differences in expression levels of beta-actin, GAPDH, and 18S rRNA. SPSS 20 (SPSS, Chicago, IL, USA) was used for analysis and a P value of <0.05 was considered statistically significant.

Results

The anthropometric, and biochemical characterizations of the participants are provided in Table 2.

Table 2. The anthropometric and biochemical data of obese and normal-weight women.

Characteristics	Controls	Obese subjects	p-value
FBG, mg/dl	89.88±2.35	86.42±1.86	0.254
TC, mg/dL	146.51±8.96	179.65±5.94	0.004
HDL-C, mg/dL	43.81±1.45	45.10±1.62	0.557
LDL-C, mg/dL	88.43±7.07	113.05±4.62	0.006
AST, U/L	16.7 (12.5-21.4)	21.1 (16.2-23.95)	0.223

ALT, U/L	12.9 (11-22.2)	21.65 (15.35-30.2)	0.406
BMI, kg/m^2	23.49 (22.86-24.34)	41.73 (36.35-46.77)	0.000
WC, cm	86 (85-89)	114 (110.25-119.25)	0.000
Hip, cm	95 (90-97)	128 (120-134)	0.000
WHR, -	0.90±0.01	0.91±0.01	0.836

FBG ,fasting blood glucose; TC, total cholesterol; HDL- C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; AST, aspartate amino transferase; ALT ,alanine amino transferase; BMI ,body mass index; WC, waist circumference; WHR ,waist-to-hip ratio;

The total RNA were retrieved from subcutaneous adipose tissue of normal-weight and obese subjects and the expression levels of beta-actin, GAPDH, and 18S rRNA were determined by by RT-qPCR. The mRNA expression levels of 18S rRNA were almost identical in two studied subjects (p=0.46). Moreover, there was no significant change in the mRNA levels of beta-actin and GAPDH in subcutaneous adipose tissue of all participant (p=0.58 and p=0.2 ,respectively) (fig. 1).

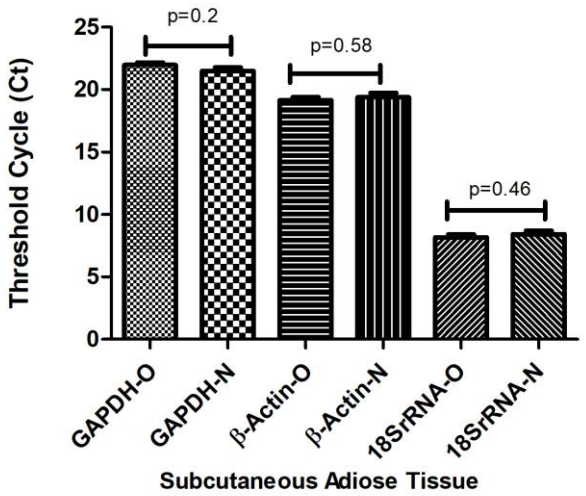

Figure 1. Expression of beta-actin, GAPDH, and 18S rRNA genes in the subcutaneous adipose tissue of obese (O) and normal-weight (N) individuals. Data was shown as mean ± standard error of mean (SEM).

Each RNA sample was consequently analyzed by RT-qPCR for determination of beta-actin, GAPDH, and 18S rRNA expression levels in visceral adipose tissue of normal-weight and obese subjects. 18S rRNA expression levels were almost identical in all studied subjects (p=0.29). The mRNA expression levels of both beta-actin and GAPDH were also similar in the visceral adipose tissue of all participants (p=0.13 and p=0.14 ,respectively) (fig. 2).

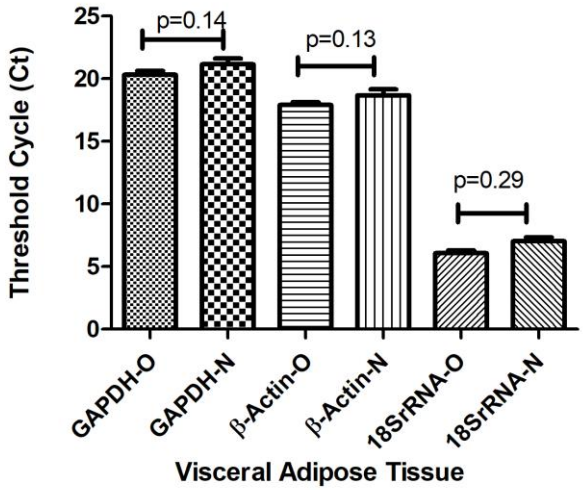

Figure 2. Expression of beta-actin, GAPDH, and 18S rRNA genes in the visceral adipose tissue of obese (O) and normal-weight (N) individuals.Data was shown as mean ± standard error of mean (SEM).

Discussion

In this study, we measured the utility of beta-actin, GAPDH, and 18S rRNA as housekeeping genes for normalization of gene expression, evaluated as mRNA level by RT-qPCR in subcutaneous and visceral adipose tissue of normal-weight and obese subjects. The average expression levels of such housekeeping genes were determined in isolated RNA of subcutaneous and visceral adipose tissue which showed no difference at the expression levels. Moreover, the present study showed the importance of confirming housekeeping genes prior to normalizing mRNA expression levels of target genes.

In molecular biological studies, gene expression measurements are one of the most commonly-used methods contributed to the recognition of different metabolic pathways. RT-qPCR is the most applicable and commonly used method for the gene expression analysis. Then, choosing a reliable reference gene is important for obtaining correct gene expression data [15]. Today, the most common used housekeeping genes are beta-actin, GAPDH, and 18S rRNA in *in vivo* and *in vitro* studies. However, some studies demonstrated that such reference genes present a significant variation in various cells, tissues and experimental situations [16, 17].

Investigations for the selection of a reliable housekeeping gene as a reference in adipose tissue have been described in many studies with conflicting results [18-20]. To the best of our knowledge, no study has reported the comparison of three well-known reference genes including beta-actin, GAPDH, and 18S rRNA in subcutaneous and visceral adipose tissue of normal-weight and obese subjects simultaneously. Therefore, in this study, we aimed to investigate whether the expression levels of beta-actin, GAPDH, and 18S rRNA are fluctuating in both subcutaneous and visceral adipose tissue of normal-weight and obese subjects using RT-qPCR for the first time.

Previous studies also reported findings from comparative analysis of the same three reference genes using RT-qPCR analysis. Although different cell types such as murine fibroblasts and human T lymphocytes were used and furthermore, their findings are inconsistent with ours, with significant variations in β-actin and GAPDH gene expression levels and only borderline modifications in 18S rRNA expression levels in both cell types [21, 22].

Moreover, several studies considered these housekeeping genes along with other types of genes in adipose tissue through different conditions with contradictory results to ours. For instance, in an investigation on the validity of 10 reference genes in adipose tissue, it was reported that our studied genes 18S rRNA, beta-actin, and GAPDH were identified as the genes with moderate or even high variations compared to other reference genes in this tissue (ranked as 6, 8, and 10, respectively). Then, it challenged the reliability of the most frequently used reference genes in adipose tissue [18].

Interestingly, in a recent study, the validation of 15 housekeeping genes including beta-actin, GAPDH, and 18S rRNA for normalization was conducted based on RT-qPCR analysis and it was indicated that despite beta-actin being often used as a reliable reference gene for this process, but it was an unstable gene in the human adipose tissue. Moreover, they stated that GAPDH expression in adipose tissue is mostly presented with a higher difference in comparison with 18S rRNA and beta-actin, which was in contrast to *in vitro* cultured human adipocytes with a stable expression of GAPDH [19, 20]. Remarkably, Catalán et al. reported that 18S rRNA is the most stable reference gene in adipose tissue regardless of the pathological conditions of obesity or obesity-related disorders. Furthermore, they indicated that GAPDH is the most variable reference gene, being overexpressed in the obese subjects [23]. In contrast, Centre et al. introduced GAPDH with a high stability in human epicardial adipose tissue [24]. Another study on three types of rat adipose tissue including epididymal white adipose tissue, inguinal beige adipose tissue, and brown adipose tissue indicated the highest stability of GAPDH in the first tissue, while beta-actin had the highest stability in the next two tissues [25].

Putting these data together, to prevent the development of results with no accurate estimations in RT-qPCR, using a housekeeping gene with no variation through cell cycle or pathological situations is required. Although there are numerous studies indicating the use of beta-actin, GAPDH, and 18S rRNA as suitable reference genes in human cells or tissues, there is still some controversy about using them in particular human tissues such as subcutaneous and visceral adipose tissue in two normal- and overweight people which we investigated here.

Conclusion

Altogether, our study indicates that normalization to all beta-actin, GAPDH, and 18S rRNA housekeeping genes gave reliable results in both subcutaneous and visceral adipose tissue when comparing normal-weight and obese subject. Then,

these housekeeping genes should therefore be the preferred reference genes for normalization when studying gene expression in human adipose tissue.

Conflict of Interest

There is no conflict of interest among authors.

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