Validation of Reference Genes for qPCR Analysis of Resistance Training and Androgenic Anabolic Steroids on Hypothalamus, Adrenal Gland and Fat Tissue

Renan Pozzi, Leandro Fernandes, Bruno FA Calegare and Vânia D’Almeida*

Department of Psychobiology, Universidade Federal de São Paulo, UNIFESP, São Paulo, Brazil

Abstract

Background: Real-time quantitative Polymerase Chain Reaction (qPCR) is a technique used for quantification of gene expression and the use of reference genes is very important to normalize the quantification results.

Aim: To validate the most suitable reference genes for resistance exercise training (REx) and use of nandrolone decanoate (DECA) in three different rat tissues.

Methods: A total of 40 adult male Wistar rats were distributed into four groups: exposed to vehicle three times per week (wk) (CT); eight wk of REx exposed to vehicle three times per wk (T); exposed to DECA three times per wk (D); eight wk of REx exposed to DECA three times per wk (TD). Stability of the following genes was evaluated: beta actin (Actb), alpha Tubulin (Tubulin), Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Hypoxanthine phosphoribosyltransferase-1 (Hprt1) and 18S Ribosomal RNA (18s) in hypothalamus, adrenal gland and mesenteric fat tissue using GeNorm, NormFinder and BestKeeper software.

Results: In hypothalamus and adrenal, all genes were suitable and none was rejected by statistical analysis; however, in fat tissue, Actb, Gapdh and Hprt1 genes were rejected by geNorm but not the others two software.

Conclusion: In hypothalamus and adrenal all selected genes analyzed were stable and can be used for qPCR gene expression analysis. However, in fat tissue we suggest the Tubulin gene as most stable gene.

Keywords: qPCR: Endogenous control gene; Resistance training; Androgenic anabolic steroids; Rats

Abbreviations: 18s: 18s Ribosomal RNA; AAS: Androgenic Anabolic Steroids; Actb: Beta Actin; Bp: Base Pair; Cq: Cycle Quantification; CT: Control Group; D: Nandrolone Group; DECA: Nandrolone Decanoate; Gapdh: Glyceraldehyde-3-phosphate Dehydrogenase; Hprt1: Phosphoribosyltransferase-1; REx: Exercise Training; qPCR: Real-time Quantitative Polymerase Chain Reaction; T: Training group; TD: Training and Nandrolone Group; Tubulin: Alpha Tubulin; Wk: Week

Introduction

Real-time quantitative chain reaction (qPCR) relative or absolute analysis requires appropriated endogenous gene as reference gene for data normalization, which are known by: housekeeping gene, normalization gene, endogenous control gene, internal reference gene and suitable reference genes [1]. The reference gene is used to normalize the target gene expression and, for this reason, the incorrect choice of reference genes can alter final results [1]. Our group has validated reference genes for rat models of sleep deprivation [2] and hypoxia [3]. To our knowledge, there are no studies concerning validation of reference genes to analyze the effects of resistance exercise (REx) and androgenic anabolic steroids (AAS) use on gene expression. A good reference gene must show minimum variation of expression in all experimental groups or, in other words, its expression should not be influenced by experimental conditions. Considering the increasing number of exercise-related and/or anabolic steroids articles, validation of the most stable reference genes for qPCR was considered of interest.

AAS are manipulated compounds derivatives of testosterone, whose main function is to isolate the anabolic effect. They are important for the treatment of growth-related diseases, osteoporosis and anemia; however, when used at supraphysiological doses, they may produce side effects such as water retention, early closing of the bone epiphysis [4], aggressiveness [5,6], irritability, hostility, cognitive symptoms such as distractibility, forgetfulness and confusion, testicular atrophy, changes in the prostate and seminal vesicles, gynecomastia, growth changes [7], development of hepatic cysts [5], cardiovascular events such as myocardial infarction, cerebral infarction and pathological hypertrophy, increasing the likelihood of arrhythmias and stroke [8,9]. Thus, despite the abovementioned risks and being prohibited in many countries, athletes and amateur practitioners use supra physiological dosages of steroids to increase the performance and free fat mass.

Resistance exercise, commonly called weight training, is a type of exercise that has as main objective muscle strength gaining. The authors suggest that REx training is a valid strategy to improve blood pressure, insulin resistance, muscle mass and reduce circulating levels of inflammatory markers [10]. Furthermore, physiological and psychological benefits of REx are considered important in physical rehabilitation and treatment programs [11]. Therefore, a number of gene expression and molecular biological studies has been conducted in REx training and steroids models, including ladder exercise models [12-15]. There is a methodological gap, in which there is a lack of studies that identify the best reference gene in these areas of knowledge.

*Corresponding author: Vânia D’Almeida, Department of Psychobiology, Universidade Federal de São Paulo, Street Botucatu, 862-1st floor-Vila Clementino, São Paulo-SP-CEP: 04023062, Brazil, Tel: 55-11-21490155; E-mail: vaniadalmeida@uol.com.br

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It is known that there is no universal reference gene although Actb, Gapdh, Hprt1 and β2M, among others are the most commonly used [16]. There are no studies regarding reference genes in this area; therefore, the aim of this study was to validate reference genes for REx and AAS use in rat hypothalamus, adrenal gland and mesenteric fat tissue.

Materials and Methods

Animals

The study was performed using 40 male Wistar rats (10-wk-old; 300-350 g) from CEDEME (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia). Animals were maintained in the Department of Psychobiology facility (Universidade Federal de São Paulo). Room temperature was 22°C (± 1) with 12:12 h light-dark cycle and access to food and water ad libitum was allowed. This study was conducted according to the Ethical of the use of Laboratory Animals Guidelines and its experimental protocol was approved by the Ethical Committee of Universidade Federal de São Paulo (#177700/2013).

Groups

A total of 40 Wistar rats (10 wk old) was distributed into four groups: exposed to vehicle (peanut oil-subcutaneous administration 1 ml/kg) 3 times/wk during 8 wk (CT); resistance exercise during 8wk and exposed to vehicle (peanut oil-subcutaneous administration 1 ml/kg) 3x/wk during 8 wk (T); exposed to DECA (subcutaneous administration 5 mg/kg) 3 times/wk during 8 wk (D); submitted to resistance exercise during 8 wk and exposed to DECA (subcutaneous administration 5 mg/kg) 3 times/wk during 8 wk (TD).

Exercise training and drug treatment

The training protocol consisted of progressive REx, 5 times/wk during 8 wk [adapted from 17-19]. A vertical ladder (110 cm high by 18 cm wide, inclined at 80º with 2 cm spacing between rungs) was used; at the top of the ladder there was a dark box (20 cm × 20 cm ×20 cm), where the animal could rest between sets (1 min). Every week, the maximum carrying loading (MCL) was tested, so that the periodization could be determined (Table 1).

Supraphysiological nandrolone decanoate doses (5 mg/kg) were injected subcutaneously to each animal 3 times/wk for 8 wk (15 mg/kg/wk). This dosage was chosen for being equivalent to that used by athletes in physical exercise [20,21]. The peanut oil was used as vehicle at the same volume of DECA (1 ml/kg).

Gene selection

Exercise training and androgenic anabolic steroids affect several systems at cellular level, thus, candidate reference genes were selected among the most common reference genes from animal models in the literature. Reference genes selected were beta actin (Actb), alpha Tubulin (Tubulin), glyceroldehyde-3-phosphate dehydrogenase (Gapdh) hypoxanthine phosphoribosyltransferase-1 (Hprt1) and 18s ribosomal RNA (18s). Primers (Table 2) were designed and synthesized by IDT (Integrated DNA Technologies - www.idtdna.com) according to published Genbank sequences.

RNA extraction, cDNA and qPCR

After 24 h of experimental issue, the animals were euthanatized by decapitation between 07:00 am to 10:00 am. Fasting for at least 2 hours was established.

The hypothalamus and adrenal gland were collected and RNA extraction was performed using TRizol Plus RNA Purification Kit (CAT#12183-555 Ambion RNA, Life Technologies). Mesenteric fat tissue was also collected and total RNA was extracted using the RNeasy Plus Universal Mini Kit (CAT#74004, QIAGEN), according to manufacturer’s specifications. RNA was pretreated with DNase I (2 U/µl), 10X DNase I Buffer (100 µM Tris- pH 7.5, 25 mM MgCl2, 5 mM CaCl2) and incubated for 37°C for 30 sec (Inverigo) according to manufacturer’s specifications. The 28S and 18s integrity of RNA was evaluated using agarose gel electrophoresis. RNA quantification was performed using spectrophotometry (NanoDrop) and purity was evaluated using two optimal wavelengths: ratio of 260/280 for nucleic acids (1.8<sample>2.2) and ratio of 260/230 for organics contaminations (1.8<sample>2.2).

cDNAs were synthesized using 1 µg of total RNA were placed in the presence of first mixture containing 0.5 µg/µl of Random Primers (Promega) and 3 mM of MgCl2, 5 mM CaCl2 and incubated for 37°C for 30 sec (Inverigo) according to manufacturer’s specifications. The 28S and 18s integrity of RNA was evaluated using agarose gel electrophoresis. RNA quantification was performed using spectrophotometry (NanoDrop) and purity was evaluated using two optimal wavelengths: ratio of 260/280 for nucleic acids (1.8<sample>2.2) and ratio of 260/230 for organics contaminations (1.8<sample>2.2).

qPCR was performed using SYBR Green PCR Master Mix (AppliedBiosystem, Warrington, UK) and StepOnePlus Real-Time PCR (AppliedBiosystem, Warrington, UK). Each reaction was performed using 2 µl of cDNA, 6 µl of H2O, 2 µl of primers (forward and reverse at 0.5 µM each) and 10 µl SYBR green PCR Master Mix to the final volume of 20 µl. All samples were analyzed in duplicates and the average values were used. Design layout was: Holding stage: 3 min at 50°C and 10 min at 95°C; Cycling stage (no of cycles: 40): 15 sec at 95°C and 30 sec at 72°C. The software geNorm uses the M-value as a stability variable, to assess the stability of selected reference genes by different methods. All software are freely available to download permanently or as a demo for free for 14 days: geNorm ([https://www.biogazelle.com/qbaseplus]; NormFinder (http://moma.dk/normfinder-software); and BestKeeper ([http://www.gene-quantification.de/bestkeeper. html#download]).

The software geNorm uses the M-value as a stability variable, directly assessing linear scale expression quantities by using the standard curve and absolute quantification. The gene with the lower value of M

| Sessions/wk | 1st Session | 2nd Session | 3rd Session | 4th Session |
|-------------|-------------|-------------|-------------|-------------|
| 1st Day     | 50          | 50          | 50          | 50          |
| 2nd Day     | 50          | 50          | 50          | 50          |
| 3rd Day     | 50          | 50          | 50          | 50          |
| 4th Day     | 50          | 50          | 50          | 50          |
| 5th Day     | 50          | 50          | 50          | 50          |
| 6th Day     | 50          | 50          | 50          | 50          |
| 7th Day     | 50          | 50          | 50          | 50          |
| 8th Day     | 50          | 50          | 50          | 50          |

Table 1: Resistance training periodization in % of MCL.
is considered the “most stable”. NormFinder also uses the values from absolute quantification to calculate stability, which indicates as Stability value the best candidate by the lower value. BestKeeper uses the HKG index which calculates the geometric average of the “most stable” reference genes by Repeated Pair-wise Correlation Analysis and p-value (p<0.05). GeNorm and NormFinder use 2^(-ΔCq) and the BestKeeper uses Cq values for analysis.

## Results

### Cycle quantification (Cq) distribution

The results related to fractional qPCR cycles are represented as follow: 1) Hypothalamus (Figure 1)-Gapdh showed the lowest standard deviation (± 0.69), followed by Actb (± 1.27), Hprt1 (± 1.35), Tubulin (± 1.69) and 18s (± 2.05); 2) Adrenal gland (Figure 2)-results showed that Hprt1 had the lowest standard deviation (± 0.96) followed by Gapdh (± 1.25), Actb (± 1.33), Tubulin (± 1.87) and 18s (± 1.92); 3) Fat tissue (Figure 3), Gapdh showed the lowest variation Cqs (± 1.52), followed by Hprt1 (± 1.65), Tubulin (± 2.26), 18s (± 2.48) and Actb (± 2.83).

### BestKeeper analysis

In hypothalamus, when comparing all experiments groups, CT vs. D, CT vs. D or CT vs. TD the most stable gene was 18s gene followed by Actb, Tubulin, Hprt1 and Gapdh genes.

For adrenal gland, 18s gene was the most stable when analyzing all groups, CT vs. D, and CT vs. T vs. D groups and the less stable was Hprt1. Rank sequence are available in Table 3.

In fat tissue when all groups were compared, as well as CT vs. T, the most stable gene was Tubulin followed by Actb, 18s, Hprt1 and Gapdh. However, when CT vs. D and CT vs. TD were compared Tubulin remains the most stable gene and Hprt1 was rejected (Table 3).

### Normfinder analysis

Analysis in Normfinder software showed that all genes in all groups were suitable, in other words, all genes showed stability values less than 0.15 [22], value considered by software. Moreover, this software showed best combination and stability value of two genes, e.g. in hypothalamus when compared all groups the best combination stability were Actb and 18s with 0.02 M-value. The other values are presented in Table 4.

In hypothalamic tissue, the Actb gene was the most stable gene when compared all groups and CT vs. D groups. Moreover, when comparing CT vs. T and CT vs. TD groups, the Hprt1 gene was considered the most stable gene. When all groups were analyzed the following rank was obtained: Actb, 18s, Hprt1, Tubulin and Gapdh genes. When

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**Table 2: Reference genes ID and primers design.**

| Gene | ID GeneBank | Forward (5’ – 3’) | Reverse (5’ – 3’) | Bp | Efficiency* | T°C | Primer [ ] |
|------|-------------|------------------|------------------|----|-------------|-----|-----------|
| Beta Actin | NM_031144.3 | GTCGTGGATTGTTGCTCTATC | CAGTCCGCTAGAAGCATT | 122 | Hypothalamus: 97.8% | 60°C | I: 10 μM F: 0.5 μM |
| Alpha Tubulin | NM_022298.1 | GACCTGGAACCCACGTATT | ATCTTCTTGCTGATGTAG | 90 | Hypothalamus: 97.3% | 60°C | I: 10 μM F: 0.5 μM |
| Gapdh | NM_017008.4 | CATGGGCTCTGGTGCCCTA | CGGACATGTCAGATCAA | 55 | Hypothalamus: 103.6% | 60°C | I: 10 μM F: 0.5 μM |
| Hprt1 | NM_012583 | GGGAAAGTGGAAGGCAAATG | GCCACATCAAACAGACTCTTGAG | 76 | Hypothalamus: 98.7% | 60°C | I: 10 μM F: 0.5 μM |
| 18s | NR_046237 | CAGAGCAGGTGACAGATGA | CAAATCGTCCACAACACTAA | 83 | Hypothalamus: 99.5% | 60°C | I: 10 μM F: 0.5 μM |

**Figure 1:** Hypothalamus quantification cycle distribution of reference genes expression from hypothalamus.

**Figure 2:** Adrenal gland quantification cycle distribution of reference genes expression from adrenal gland tissue.
comparing CT vs. D groups, Actb, followed by 18s, Tubulin, Hprt1 and Gapdh genes. At CT vs. T and CT vs. TD groups, Hprt1, followed by 18s, Tubulin, Gapdh and Actb genes.

At adrenal gland, the ranks considering all groups and CT vs. TD groups were: 18s, followed by Tubulin, Gapdh, Actb and Hprt1 genes; for the CT vs. T groups: Tubulin, followed by Actb, 18s, Gapdh and Hprt1 genes; and for the CT vs. D groups: Tubulin, followed by 18s, Gapdh, Hprt1 and Actb genes.

The Tubulin gene was considered the most stable gene for fat tissue in all moments and Gapdh was the less stable gene, except in CT vs. D the Hprt1 gene was the less suitable gene (Table 4).

**GeNorm analysis**

Analysis made by software showed that in hypothalamus and adrenal gland all candidates are stable and could be used as reference genes but in mesenteric fat tissue, only Tubulin gene was stable in all analysis. The others genes (Hprt1, Actb, 18s and Gapdh genes) had a 1.5 M-value and it, was not considered good reference genes at least one time.

In hypothalamus the most stable gene was Actb when comparing all groups, CT vs. D and CT vs. TD groups, followed by Hprt1, Tubulin, Gapdh and 18s genes; and for the CT vs. T groups: Actb, followed by Hprt1, Gapdh, Tubulin and 18s genes.

In adrenal gland, it was observed that the Hprt1 gene had the lower rank in all analysis. In all groups and CT vs. TD groups the Actb gene had the higher rank, following by Gapdh, Tubulin, 18s and Hprt1 genes. For the CT vs. T groups, Gapdh was the most stable gene, followed by Actb, Tubulin, 18s and Hprt1 genes; and for the CT vs. D, Gapdh was most suitable gene followed by Actb, 18s, Tubulin and Hprt1 genes.

Considering the mesenteric fat tissue the unique stable gene was the Tubulin in all analyzes. Hprt1 was stable when all groups and CT vs. T were compared. The other genes exceeded the M-value allowed by the software (Table 5).

**Discussion**

To determination of relative expression of target gene in any assay there is a necessity of use a reference gene. Accordingly, the reference gene is usually an endogenous gene in which expression is unchanged regardless of intervention [23]. The use of reference genes is appropriated only if they are tested, normalized and considered stable, some authors believe that it is wrong any gene as reference gene without validating their suitability before running the experiment [24]. In this study, this amount of genes gives us an overview of what can be used in the experiments using AAS and RX.

A simple way to find the most stable reference gene is to analyze the Cq variation and use the one with the lowest variation between experimental groups [25]. However, there are specific software for this type of analysis; the most used in the literature are BestKeeper, Normfinder and geNorm [24]. To our knowledge, there are no studies investigating the most stable genes regarding exercise and anabolic steroids use; therefore, our study will contribute to a more adequate choice of reference genes for those experiments. We separate the analysis group to group to have a more complete analysis, so analyze all groups and then each separate factor, so there is an interpretation according to each intervention.

The analysis of more than one reference gene has been shown to be useful to validate the data, as well as to confirm the results. The authors suggest the use of three reference genes; if the results with the first

| Tissue          | Hypothalamus | Adrenal gland | Mesenteric fat |
|-----------------|--------------|---------------|----------------|
| Groups          | CT, D, T and TD | CT, D, T and TD | CT, D, T and TD |
| Gene            | Actb, Tubulin, 18s, Gapdh, Hprt1 | Actb, 18s, Gapdh, Hprt1 | Actb, 18s, Gapdh, Hprt1 |
| r               | 0.96, 0.93, 0.91, 0.89, 0.77 | 0.98, 0.97, 0.96, 0.95, 0.86 | 0.98, 0.95, 0.89, 0.86, 0.82 |
| p-value         | 0.001, 0.001, 0.001, 0.001 | 0.001, 0.001, 0.001, 0.001 | 0.001, 0.001, 0.001, 0.001 |

**Table 3: Ranking of reference genes by Bestkeeper software analysis.**
We conducted a search in PubMed and selected 20 articles that used qPCR analysis of gene expression in the hypothalamus, adrenal gland, and mesenteric fat tissue to evaluate the effects of anabolic androgenic steroids on gene expression. The results of the reference gene study are controversial, and some authors did not find stable results for Actb and Gapdh genes in injured muscle, the results were rejected by geNorm and BestKeeper [44]. In other studies, no stable results for Actb and Gapdh genes were rejected in muscle tissue by qBase, software that uses M-Value to analyze reference genes [45]. In our study, Actb and Gapdh genes were shown to be stable in all tissues analyzed by three different software; the only exception was the hypothesis test for mesenteric fat analysis through geNorm, which rejected both genes, along with Hprt1.

In hypothalamus and adrenal gland, the three software used in our study showed similar results using different analyses (all groups together or separately, as in CT vs. T, CT vs. D or CT vs. TD). Many types of exercise and/or AAS could alter gene expression in different ways but, in most of our analyses, the values and ranking of genes were similar in all experimental groups. None of the genes were rejected by the two different reference gene methods must be used, and, if they are similar, it is not necessary to evaluate a third gene [26]. Dheda et al. [27] demonstrated after three experiments with different reference genes that the results can be significantly different from those obtained when an invalid reference gene is used. This incorrect choice, therefore, results may be erroneous. The same authors also suggest strongly supporting the argument for validation of reference genes prior to their use.

In order to verify if there were common reference genes in these studies, we conducted a search in PubMed and selected 20 articles that demonstrated after three experiments with different reference genes that the results can be significantly different from those obtained when an invalid reference gene is used. This incorrect choice, therefore, results may be erroneous. The same authors also suggest strongly supporting the argument for validation of reference genes prior to their use.

### Table 4: Ranking of reference genes by the NormFinder software analysis.
```
| Tissue | Hypothalamus | Adrenal gland | Mesenteric fat |
|--------|--------------|---------------|----------------|
| Groups | CT, D, T and TD | CT, D, T and TD | CT, D, T and TD |
| Genes  | Actb, Hprt1, Tubulin, Gapdh, 18s | Actb, Gapdh, Tubulin, 18s | Actb, Gapdh, 18s |
| M-Value| 0.95, 1.02, 1.23, 1.26 | 0.75, 0.77, 0.88, 0.99 | 1.21, 1.39, 1.50,* 1.79,* |
| Groups | CT vs. T | CT vs. T | CT vs. T |
| Genes  | Actb, Hprt1, Gapdh, Tubulin, 18s | Actb, Tubulin, 18s | Actb, 18s |
| M-Value| 0.93, 0.94, 1.14, 1.22, 1.35 | 1.32, 1.50,* 1.69,* 1.79,* | 2.01,* |
| Groups | CT vs. D | CT vs. D | CT vs. D |
| Genes  | Actb, Hprt1, Tubulin, 18s | Actb, Gapdh, 18s | Actb, Gapdh, 18s |
| M-Value| 0.96, 1.14, 1.14, 1.16, 1.36 | 1.25, 1.44, 1.57,* 1.63,* 2.03,* |
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### Table 5: Ranking of reference genes by GeNorm software analysis.
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| Tissue | Hypothalamus | Adrenal gland | Mesenteric fat |
|--------|--------------|---------------|----------------|
| Groups | CT, D, T and TD | CT, D, T and TD | CT, D, T and TD |
| Genes  | Actb, Hprt1, Tubulin, Gapdh, 18s | Actb, Gapdh, Tubulin, 18s | Actb, Gapdh, 18s |
| M-Value| 0.92, 0.95, 1.01, 1.04, 1.21 | 0.84, 0.89, 0.95, 1.02, 1.10 | 1.34, 1.60,* 1.72,* 1.77,* 2.07,* |
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Data expressed as M-Value. Lowest M-value indicates most stable gene. Best Comb. (Best combination of two genes); CT: Control Group; D: DECA group; T: Training Group; TD: Training and DECA Exposed Group.

*Result above the limit considered adequate by software analysis.

Data expressed as Stability value. Lowest Stability value indicates most stable gene. Best Comb. (Best combination of two genes); CT: Control Group; D: DECA group; T: Training Group; TD: Training and DECA Exposed Group.

*Result above the limit considered adequate by software analysis.
any of the software; therefore, they are all suitable reference genes for qPCR analysis in rat hypothalamus and adrenal gland.

In mesenteric fat tissue, however, there were some discrepancies between results. All genes were stable and considered suitable as reference genes by Normfinder, but not by Bestkeeper and geNorm analysis. GeNorm shows that the only stability gene was Tubulin (in all analysis) and when used Bestkeeper software, the only exception was Hprt1 gene, showed above the limit considered adequate.

All software used to check the stability of genes are validated and considered replicated. Most often, results are repeatable despite using different calculations, as M-value and pair-wise correlation. Whereas two of the three software considered all stable candidates and only geNorm considered only Tubulin as stable, we suggest that the best gene to be used of adipose tissue is indeed Tubulin, however, could be used the other candidates for qPCR analysis if confirm with other reference gene.

It is also valid to emphasize that when we analyze different groups with different interventions, the software will also modify the results. When the NormFinder was used for each analysis groups, the software put genes in a different position and we showed the best combination.

In our study the three software utilized in these analyses produced similar results but the order of the results was not identical and in some cases was considerably different, which corroborate the findings reported by other study [46]. This difference may be attributed to different mathematical models used in each program [24]. On the other hand, there was found similar results for the software geNorm and NormFinder but not for BestKeeper, this can be justified by the fact that this software used the Pearson correlation method to classify the reference genes, a different method compared to the others software [3].

This study is the first to validate reference genes for the evaluation of REx and AAS use effects in different rat tissues. It is important to note that there is no ideal universal reference gene. This work can help you find good candidates, though, for each experiment, species, tissue and other conditions, it is necessary to perform and confirm a specific validation of reference genes, in order to analyze gene expression results more adequately.

Conclusion
In conclusion, our results do not suggest a specific reference gene for hypothalamus and adrenal gland, since all genes analyzed (Actb, 18s, Hprt1, Tubulin and Gapdh) were stable and suitable for gene expression normalization through qPCR. However, in mesenteric fat tissue, the only suitable reference gene accepted by three software was Tubulin gene.

Competing Interests
None of the authors has any conflict of interest in submitting this manuscript.

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