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Validation of reference genes for RT-qPCR in cardiac tissue of rats induced to obesity and diabetes

Validação dos genes de referência para RT-qPCR em tecido cardíaco de ratos induzidos à obesidade e ao diabetes

Validación de los genes de referencia para RT-qPCR en tejido cardíaco de ratón inducido por obesidad y diabetes

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Abstract

The validation of reference genes is an essential step for any RT-qPCR analysis. In this way, the present paper aimed to identify and validate reference genes for RT-qPCR in cardiac

tissue of rats of the Rattus norvegicus albinus specie, submitted to obesity associated or not to type 2 diabetes mellitus. For this, the metabolic changes were induced at the 42nd day of life and the euthanasia was performed on the 70th day. The heart apexes were collected and destinates for RNA extraction. The RT-qPCR technique was performed in own thermocycler, the efficiency of the primers found by the LinReg software and the stability of the expression of the reference genes in the samples was analyzed by the RefFinder algorithm. The candidates for reference genes were GAPDH, POLR2A, RPL32, and RPL4 and the target gene used to verify the differences in gene expression of candidates for reference genes was CMA1. The obese animals showed a decrease in CMA1 gene expression when compared to the two most stable reference genes. The opposite occurs when it is compared to the two less stable reference genes. The GAPDH and POLR2A genes are the best to normalize the reactions with the samples in question. There is no universal reference gene for all situations, which requires systematic validation for each situation. The use of unvalidated reference genes may compromise the interpretation of the expression of the target genes, which would prevent the reflection of the actual situation.

Keywords: Heart; Rodend; Normalization; RefFinde; GAPDH.

Resumo

A validação dos genes de referência é uma etapa essencial para qualquer análise de RT-qPCR. Dessa forma, o presente trabalho teve como objetivo identificar e validar genes de referência para RT-qPCR em tecido cardíaco de ratos da espécie Rattus norvegicus albinus, submetidos à obesidade associada ou não ao diabetes mellitus tipo 2. Para isso, as alterações metabólicas foram induzidas no 42º dia de vida e a eutanásia foi realizada no 70º dia. Os ápices cardíacos foram coletados e destinam-se à extração de RNA. A técnica de RT-qPCR foi realizada em termociclador próprio, a eficiência dos primers encontrados pelo software LinReg e a estabilidade da expressão dos genes de referência nas amostras foram analisadas pelo algorítmo RefFinder. Os candidatos a genes de referência foram GAPDH, POLR2A, RPL32 e RPL4 e o gene alvo usado para verificar as diferenças na expressão gênica dos candidatos a genes de referência foi CMA1. Os animais obesos apresentaram uma diminuição na expressão do gene CMA1 quando comparados aos dois genes de referência mais estáveis. O oposto ocorre quando comparado aos dois genes de referência menos estáveis. Os genes GAPDH e POLR2A são os melhores para normalizar as reações com as amostras em questão. Não há gene de referência universal para todas as situações, o que requer validação sistemática para

cada situação. O uso de genes de referência não validados pode comprometer a interpretação da expressão dos genes alvo, o que impediria a reflexão da situação real.

Palavras-chave: Coração; Roedor; Normalização; RefFinde; GAPDH.

Resumen

La validación de genes de referencia es un paso fundamental para análisis de RT-qPCR. El presente trabajo tuvo como objetivo identificar y validar genes de referencia para RT-qPCR en tejido cardíaco de ratas de la especie Rattus norvegicus albinus, sometidas a obesidad asociada o no a diabetes mellitus tipo 2. Fueran introducidos los cambios metabólicos a los 42 días de vida y se realizó la eutanasia a los 70 días. Los ápices del corazón fueran destinados a la extracción de RNA. La técnica RT-qPCR se realizó en termociclador propio, la eficiencia de los cebadores encontrados por el software LinReg y la estabilidad de la expresión de los genes de referencia en las muestras se analizó mediante el algoritmo RefFinder. Los candidatos a genes de referencia fueron GAPDH, POLR2A, RPL32 y RPL4, y el gen diana utilizado para verificar las diferencias en la expresión génica de los candidatos a genes de referencia fue CMA1. Los animales obesos mostraron una disminución en la expresión del gen CMA1 en comparación con los dos genes de referencia más estables. Lo contrario ocurre cuando se compara con los dos genes de referencia menos estables. Los genes GAPDH y POLR2A son los mejores para normalizar las reacciones con las muestras en cuestión. No existe un gen de referencia universal, lo que requiere una validación sistemática para cada situación. El uso de genes de referencia no validados puede comprometer la interpretación de la expresión de los genes diana, lo que evitaría el reflejo de la situación real.

Palabras clave: Corazón; Rodend; Normalización; RefFinde; GAPDH.

1. Introduction

The obesity and diabetes are multifactorial diseases that can cause damage to other organic systems, such as the renal and cardiac (Cefalu et al., 2015). In diabetes, the hyperglycemia, resulting from the deficiency of insulin production or the resistance to it, generates the formation of reactive oxygen species (ROS), and the chronic exposure to ROS may increase cardiomyocyte apoptosis and accumulation of fibrous tissue in the heart (Shuckla et al., 2017). In this context, there is an enzyme named chymase, that may be involved in this heart condition, considering that it participates in 80% of cardiac angiotensin II synthesis (Ahmad et al., 2014)

The *Rattus norvegicus albinus* specie is one of the most widely used animal models in experiments with metabolic alterations (Hunt et al., 2017), that can cause serious heart problems, and it is important to assess the timing of these changes. The alteration of the gene expression of certain genes may precede the histological or clinical changes in these animals (Khalilpourfarshbafi et al., 2017) and to verificate this event, it is necessary to evaluate the expression of the genes, which can be done by quantifying the messenger RNA (mRNA) of a given tissue, in a given time, and specific condition, through the real-time quantitative polymerase chain reaction (RT- qPCR) (Livramento et al., 2018).

Although there is a need for systematic selection and validation of reference genes for each RT-qPCR study, there is still little information available on the stability of reference genes in murine model. It is known that the stability of gene expression may vary, depending on the metabolic conditions and tissue type studied (Chen, 2017) and the increase of the number of validation studies of reference genes promises greater reliability and accuracy in studies in molecular biology, as well as better representation of reality in in vitro analysis (Rajkumar et al., 2015).

Many factors may influence the normalization of RT-qPCR, such as RNA quality and integrity and the efficiency of complementary DNA (cDNA) synthesis (Tan et al., 2012). In order to minimize these influences, standardization of reference gene expression is employed, considering that both the reference gene and target genes can be quantified in the same sample (Zhu et al., 2014). The reference genes are selected by choosing genes that maintain their constant expression in a given situation. For this, RefFinder (East Carolina University, Greenville, NC, USA) software is the most commonly used reference gene selection tool and is based on four other algorithms: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2010) and Comparative ΔCt (Silver et al., 2006), forming a final ranking. This yields more appropriate and consistent results for the RT-qPCR techniques (Livramento et al., 2018). The RT-qPCR technique is a fast and cost-effective method for assaying specific gene expression compared to other methods, such as whole transcriptome sequencing and is, thus, widely use in the cardiac field. The objective of the present study was to select reference genes with stable expression in samples of cardiac tissue of *Rattus norvegicus albinus* submitted to obesity by the hyperlipid diet and diabetes.

2. Material and Methods

This research paper is a qualitative approach of a controlled laboratory research type (Pereira et al., 2018).

2.1 Biological samples

The biological samples were obtained from rats of the specie *Rattus norvegicus albinus*, that were kept in the vivarium, under the approval of the Committee for Ethics in the Use of Animals in the Federal University of Lavras, 063/2017, from September to December 2017. Eighteen animals were equally divided into 3 groups, which were: obese animals (A), obese and diabetic animals (B) and control animals (C). All animals were isolated, individually, in metabolic cages arranged vertically in 4 cages and horizontally in 6 cages. The room had it temperature monitored, with a variation of 22ºC ± 2oC, with cycles of 12/12 hours light/dark and humidity of 45 ± 15%. The animals were induced to diabetes on the 42nd day of the experiment by using streptozotocin in a single dose of 45mg/kg by intraperitoneal route. The diabetes was proven by blood glucose measurement 72 hours after induction, when the blood was obtained by a small cut of the tip of the tail and blood glucose greater than 300mg/dL confirmed the diabetes (Lerco et al., 2003). For the induction of obesity, the animals ate a high-fat diet (Panveloski-Costa et al., 2011) (commercial feed brand Quimtia®, which contains ground whole corn, soybean meal, wheat bran, calcium carbonate, dicalcium phosphate, sodium, vitamin A, vitamin D3, vitamin E, vitamin K3, vitamin B1, vitamin B2, vitamin B6, vitamin B12, niacin, calcium pantothenate, folic acid, biotin, choline chloride, iron sulfate, manganese monoxide, zinc oxide, copper sulphate, calcium iodate, sodium selenite, cobalt sulphate, lysine, methionine, BHT, with addition of 20% swine fat for animals of groups D and E) from the first day of experiment, to reach the obesity at the 42nd day. All animals were recieving 40g of feed per day and obesity was proven through the Lee Index analysis (Panveloski-Costa et al., 2011). The euthanasia was performed on the 70th day of the experiment, when the animals were anesthetized with isoflurane by inhalation and euthanized in guillotine. The heart was collected, the cardiac apices were collected and frozen in liquid nitrogen to be assigned to the molecular biology technique, while the base of this organ was intended for histological tests.

2.2 RNA extraction and cDNA confection

The cardiac tissues were individually macerated in the presence of liquid nitrogen and subsequently vortexed with TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) to initiate RNA extraction from the samples. After centrifugation, the supernatant was collected, the isopropyl alcohol was added and the samples were kept in freezer, being centrifuged again after an hour for the RNA pellet formation. This pellet was washed with 75% ethyl alcohol and dried in a concentrator, and then, the integrity of the RNA was determined by 0.8% agarose gel electrophoresis with TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8.0), labeled with ethidium bromide. The intensity of the RNA bands after electrophoresis and the absence of blots confirm the integrity of the RNA and the RNA concentration of each sample was measured by the Nanodrop 1000 Spectophotometer (ThermoFisher Scientific, Waltham, MA, USA), being the purity of the RNA measured by the 260/230nm ratio, with desired values between 1.8 and 2.0 (Sambrook and Russel, 2002).

Subsequently, the RNA samples were treated with DNase (TURBO^{TM} DNase-Ambion, Waltham, MA, USA) according to the manufacturer's recommendations. At this phase of the experiment, a conventional PCR is performed to ensure that all DNA has been eliminated from the sample and there is only RNA from now on. The cDNA was synthesized from 800ng of total RNA for each sample using the High-Capacity cDNA Reverse Transcription Kit (Aplied Biosystems-Life Technologies, Waltham, MA, USA), according to the manufacturer's recommendation and its quality was confirmed by the *RPL32* and *GAPDH* gene, visualized by 0.8% agarose gel electrophoresis using 10μL of PCR product. The synthesized cDNA was stored at -80°C for subsequent application of the RT-qPCR technique.

2.3 Selection of reference genes and primer design

Two candidate gene reference genes were selected according to the best results previously published (Brattelid et al., 2010), ribosomal protein L32 (*RPL32*) and RNA polymerase II subunit A (*POLR2A*). Another two were choosen because of their widly use in molecular biology routine, being ribosomal protein L4 (*RPL4*) and glyceraldehyde-6-phosphate dehydrogenase (*GAPDH*),

The genetic sequences homologous to the selected genes were worked by the Basic Local Alignment Search Tool (BLAST) software, using the sequences available from GenBank (http://www.ncbi.nlm.nih.gov/). The sequences were submitted to OligPerfect and

OligoAnalyse software (Sigma-Aldrich) for primer design. The length of the primers was between 19 and 21 bp with GC content ranging from 45% to 60% and a melting temperature (Tm value) in a range from 57 to 63 C.

The specificity of the chosen primer pair was verified by the melting curve. Both the amplification efficiency and the determination coefficient (R^2) were determined during primer validation, according to the LinReg software, using a dilution containing all cDNA samples. The specifications of genes and primers are shown in Table 1.

Table 1. Description of the reference genes candidates and CMA1 gene for the techniques of RT-qPCR.

Gene	Number of access	Primers sequences (Fw/Rv 5'-3')	*Tm* (°C)	*Amplicon* (pb)	E (%)	R^2
GAPDH	NP_058704.1	GCCCAGCAAGGATACTGAGA	60.36	103	85	0,997
		GGATGGAATTGTGAGGGAGA	59.86			
RPL32	NM_013226.2	GCCCAAGATCGTCAAAAAGA	60.19	120	92	0,998
		AATCTTCTCCGCACCCTGTT	61.02			
RPL4	BC081801.1	GCCAAATCGGAGAAGATTGT	59.13	108	92	0,979
		TGCAGGCTTCTTCAGCTTCT	60.42			
POLR2A	XM_001079162.5	TCTCCCACTTCTCCTGGCTA	59.94	87	95	0,991
		CTCCTCATCGCTGTCTTCTG	58.70			
CMA1	NC_005100.4	GTGTCTGCTGCTCCTTCTCC	60.56	81	81	0,977
		CGGGAGTGTGGTATGCACT	60.85			

Source: from the authors (2020)

The described genes above are glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L32 (*RPL32*), ribosomal protein L4 (*RPL4*), RNA polymerase II subunit A (*POLR2A*), and chymase enzyme (*CMA1*).

2.4 The amplification on RT-qPCR

The technique was performed on Aplied Biosystems 7500 Real-Time PCR, with a reaction containing 5µL of SYBR®Green PCR Master Mix (Aplied Biosystems, Foster City, CA, USA) and 800ng and cDNA, with optimized concentrations of the primers (10ng/µL) and RNase free water, totalizing a volume of 10µL. Amplification conditions were as follows: 95°C for 10 minutes and thereafter 40 cycles for denaturation, at 95°C for 15 seconds, annealing and extension (performed at 60°C for 1 minute). To confirm the specificity of the primers, the melting curves were established after 40 cycles of amplification and the

temperature increase from 60 to 95°C. All RT-qPCR techniques were performed with technical and biological triplicates.

2.5 Analysis of the expression stability of the reference genes candidates

The expression levels and variations of the tested genes were determined based on the quantification of the cycles (Cq) at the cardiac tissue of rats under the following conditions: obese; obese and diabetic; control or healthy animals; and a pool of all samples. The values of Cq were determined using software 7500, version 2.0.5 (Aplied Biosystems, Foster City, CA, USA) and corrected according to the efficiency of each primer pair, and the RefFinder tool was used to access the stability of gene expression in cardiac tissue samples.

The RefFinder tool (http://150.216.56.64/referencegene.php) was employed to assess the gene expression stability in four sample sets: (i) obese rats; (ii) obese and diabetic rats; (iii) control animals; and (iv) pool of biological samples representing all sample types.

2.6 Determination of the minimum required number of the reference genes

Based on the ranking given by the RefFinder analysis, the variations were calculated for each dataset, aiming to establish the minimum number of reference genes to increase the accuracy of gene normalization. The formula Vn/n + 1 calculate the two consecutive normalization factors (FN), starting with the relative expression of values from two more stable genes, as shown by RefFinder. The calculation consists on the ratios of the log-transformed NFs of each sequential combination of two NFs and the standard deviation of the analysis data for each combination of FN is calculated too. After that, a map is drawn to show the modifications of the stability in the expression of the FN in comparison to the number of genes employed.

2.7 Validation of the reference gene by the expression of *CMA1*

To verify how the expression of a target gene is affected by the reference genes the two more stable and unstable reference genes were used to compare their expression with the expression of *CMA1* gene. The same cDNA samples were analyzed by RT-qPCR with the primers that correspond to this target gene.

3. Results

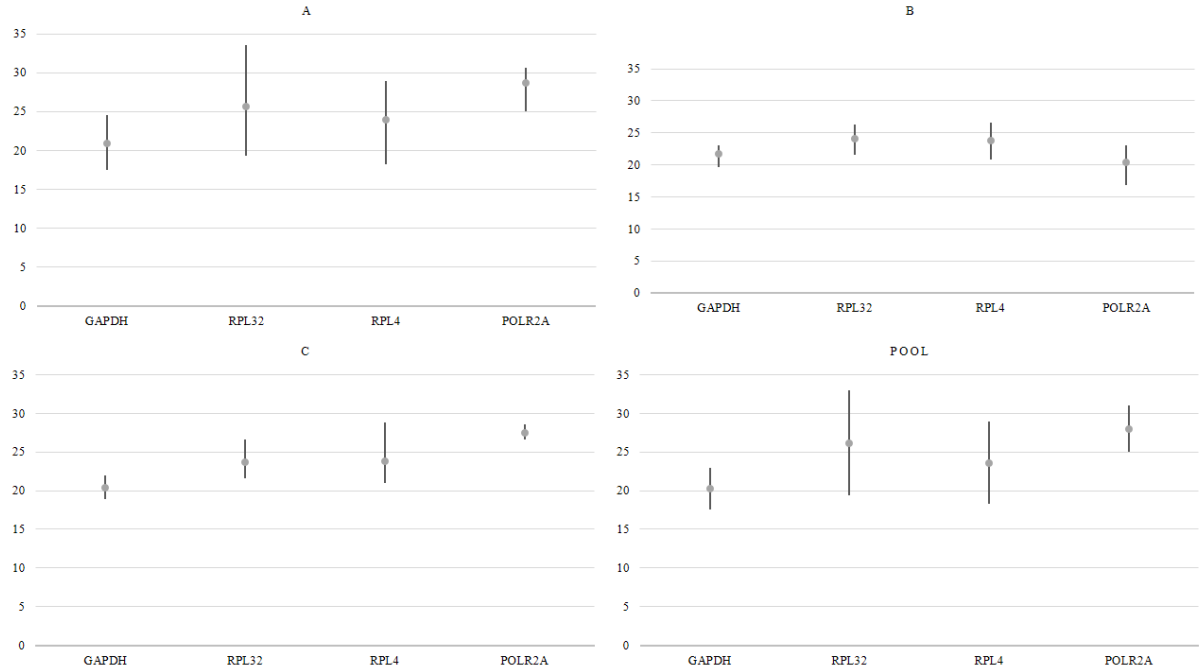
3.1 Specificity and eficiency of the primers

The specificity during the amplifications in the RT-qPCR reactions was confirmed by the presence of a single peak in the melting curve and the efficiency (E) of the RT-qPCR reaction and the determination coefficient (R^2) were calculated using the LinReg software for each primer pair. The E values ranged from 81% to 95% and R^2 values were higher than 0.97, indicating that the cDNA model was successfully duplicated at the end of each cycle.

3.2 Expression and stability of the reference genes candidates

The expression of the reference genes candidates are shown in Figure 1, with the variation, expressed in boxplot diagrams.

Figure 1. Expression of reference gene candidates determined by quantification of cycles (Cq) in sample groups. The bars indicate the maximum and minimum values of the Cqs while the circles represent the mean values. “A” means obese group; “B” means obese and diabetic group; “C” means control group; and “Pool” means samples pool.



Source: from the authors (2020).

In the analysis of the gene expression stability of reference gene candidates in the pool of samples analyzed by geNorm, the most stable genes were *POLR2A* and *GAPDH* (M = 0.968) and the least stable was *RPL32* (M = 1.651). NormFinder identified *GAPDH* (SV = 0.834) as the most stable and *RPL32* (SV = 1.52) as the least stable. The BestKeeper indicated the *POLR2A* as the most stable reference gene (SD = 1.578) and *RPL4* (SD = 2.492) as less stable. The ΔCt method determined that *GAPDH* was the most stable gene while *RPL32* was the least stable, with values of 1.493 and 1.831, respectively. The final RefFinder ranking suggests that the most stable gene was *GAPDH*, with a stability value equal to 1.189, and the most unstable, *RPL32*, with a stability value equal to 3.772.

In obese animals, compared to healthy, *GAPDH* was the candidate for the most stable reference gene for all analysis (NormFinder, geNorm, Comparative ΔCt and BestKeeper). Therefore, the final ranking by RefFinder showed that the same gene had higher stability (1.189), followed by *POLR2A* (1.861), *RPL4* (2.913), and *RPL32* (3.722) as the most unstable gene.

In obese and diabetic animals compared to healthy animals the analysis of Comparative ΔCt and NormFinder revealed that *RPL32* was the most stable gene (stability value = 1.171 and SV = 0.534, respectively). The BestKeeper and geNorm showed that the *POLR2A* gene was the most stable (SD = 1.048 and M = 1.035, respectively), and the second algorithm resulted in a tie between *POLR2A* and *GAPDH*. The final ranking by RefFinder revealed that *GAPDH* was the most stable gene (1.682), followed by *RPL32* (1.732), *POLR2A* (1.732) and *RPL4* (4.000). All of these data are set forth in Table 2 and boxplot diagrams with variation levels of reference gene candidates are shown in Figure 1.

Table 2. *Ranking* of the reference gene candidates according to the values of stability evaluated in differents sample groups.

Gene	geNorm		NormFinder		BestKeeper		Δ-Ct		RefFinder	
	M-Value	Ranking	SV-Value	Ranking	SD-Value	Ranking	ΔCt-Value	Ranking	OSV	Ranking
A x C										
GAPDH	0.986	1	1.033	1	1.535	1	1.892	1	1.000	1
POLR2A	0.986	2	1.597	3	1.634	2	2.087	2	1.861	2
RPL32	2.144	4	2.068	4	3.312	4	2.448	4	3.722	4
RPL4	1.840	3	1.421	2	3.264	3	2.148	3	2.913	3
B x C										
GAPDH	1.035	1	0.773	2	1.100	2	1.294	2	1.682	1
POLR2A	1.035	2	1.203	3	1.048	1	1.479	3	1.732	3
RPL32	1.156	3	0.534	1	2.023	4	1.171	1	1.732	2
RPL4	1.394	4	1.465	4	1.630	3	1.631	4	4.000	4
A x B										
GAPDH	1.267	1	0.747	2	1.750	2	1.772	2	1.682	2
POLR2A	1.531	3	1.837	3	1.611	1	2.180	3	2.280	3
RPL32	2.037	4	2.358	4	3.000	4	2.543	4	4.000	4
RPL4	1.267	2	0.634	1	2.167	3	1.654	1	1.316	1
Pool										
GAPDH	1.064	1	0.881	1	1.401	1	1.659	1	1.000	1
POLR2A	1.064	2	1.465	3	1.452	2	1.871	3	2.060	2
RPL32	1.856	4	1.765	4	2.566	4	2.091	4	4.000	4
RPL4	1.621	3	1.127	2	2.516	3	1.803	2	2.449	3

Source: from the authors (2020)

Samples of the cardiac tissue of each group of animals with metabolic disturbance. The genes *GAPD own thermocyclerH*, *POLR2A*, *RPL32*, and *RPL4* are indicated in the table. M-value: mean variation of expression of a given gene over the others tested; SV-value: expression stability values; DP-value: standard deviation value; ΔCt-value: value of difference between the largest and the least Cq of the samples; OSV: overall stability value, value of the final ranking, given by RefFinder.

3.3 Determination of the optimum number of reference genes

For the gene expression analysis of obese animals compared to healthy ones, the standard deviation of the values was lower for the V2/3 variation, being 0.47 compared to 0.63 of the V3/4 variation. Considering the group of obese animals compared to the obese and diabetic ones, the standard deviation of the V2/3 variation values was higher, being 0.49 compared to 0.40 of the V3/4 variation. Considering the group of obese and diabetic animals compared to healthy ones the standard deviation of the V2/3 variation values was lower, being 0.15 compared to 0.53 of the V3/4 variation. In this study the results showed that the standard deviation of the values for V2/3 variation was the lowest, being 0.40 compared to 0.52 of V3/4 variation in the samples pool. All these data are shown in Table 3.

Table 3. Standard deviation of the stability of the reference genes to determine the optimal number of reference genes for each sample group. The better values for the variations are in the cells highlighted in gray.

Variation	Sample Groups			
	A+C	**A+B**	**B+C**	**A+B+C**
V2/3	0.4723	0.4927	0.1472	0.4041
V3/4	0.6309	0.4085	0.5285	0.5248

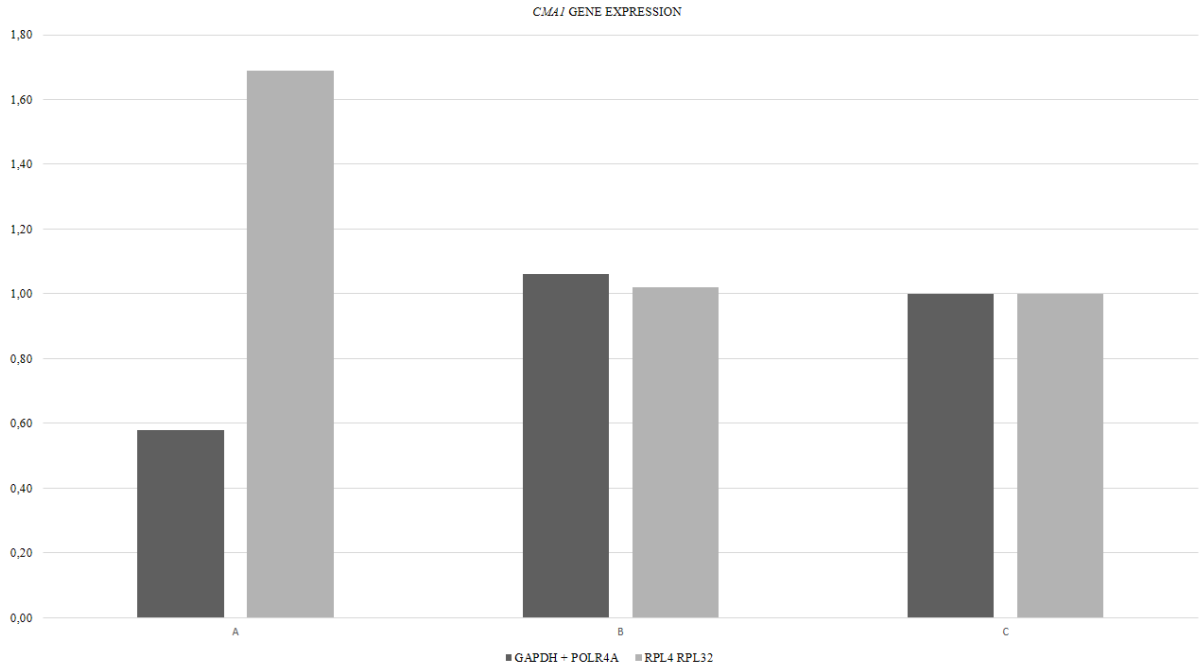
Source: from the authors (2020)

It is important to note that the standard deviation of the V2/3 contains most of the samples, indicating that, for this type of study, the optimal number of reference genes for normalization is two. The "A + C" sample correspond to obese compared to the control group; the "A + B" sample correspond to obese compared to obese and diabetic; the "B + C" sample correspond to obese and diabetic compared to control; and the "A + B+ C" sample correspond to the sample pool.

3.4 Validation of the selected reference genes

The *CMA1* gene is expressed when occurs cardiac fibrosis, situation to which obese and diabetic animals are subjected. In order to verify the impact of the selection of reference genes on the gene expression of the *CMA1* gene, two strategies were employed: the combination of the two more stable reference genes with the two less stable, in the samples pool. The expression was dependent on the reference genes used for normalization and *CMA1* levels were overestimated when reference genes not suitable for normalization were used. This gene expression pattern was stable in the relative quantification in the groups of obese and diabetic and in the control animals, with values of expression close to one. In obese animals, the relative quantification of the most stable genes was 0.58 whereas the most unstable genes were 1.69. These data are shown in Figure 2.

Figure 2. Differences in the gene expression of *CMA1* using the selected reference genes. Quantification of relative gene expression was done with two strategies: the combination of the two most stable reference genes *GAPDH* + *POLR2A* and the two less stable genes *RPL4* and *RPL32*. The columns represent the gene expression of the two combinations in each treatment. being: "A", obese; "B", obese and diabetic; and "C", control, and the relative expression refers to the standard deviation of the differences between the largest and the smallest Cq of the samples.



Source: from the authors (2020).

In the Figure 2, it is important to note that, specially for obese animals, according to the choosed reference gene, a great difference regarding the expression of the *CMA1* gene is shown. This corroborates with our initial hypothesis that the reference gene should be tested before the final analysis with RT-qPCR, because the random or commonplace choice of it culminate in an over or underestimation of the quantification of the main gene under study.

4. Discussion

Regardless of the application, experiments with RT-qPCR should be normalized with more than one reference gene, that should be validated considering its stability and aiming to avoid errors and variations within the expression analysis. The results of this work suggest that the most stable gene is *GAPDH*, followed by *POLR2A*, *RPL4*, and *RPL32* in the cardiac tissue samples pool from rats under different metabolic disturbes. These two genes were considered the most stable reference genes in rats with severe cardiac abnormalities, such as hypoxia (Brattelid et al., 2010).

Although the *GAPDH* gene is commonly used as a reference gene for RT-qPCR in murine models, its stability varies (Eissa et al., 2017). This was also evidenced in the present research, depending on the algorithm used and the metabolic conditions which the animal was submitted to. Eissa et al. (2017) evaluated other genes, such as *TBP* (tata box protein) and *RPLP0* (ribossomal protein LP0), concluding that they were more appropriate for the normalization of the reaction in rat intestinal healthy tissue. For the cardiac tissue of rats submitted to intermittent hypoxia, *GAPDH* was the most stable gene, followed by *ACTB* (actin β) (Julian et al., 2016). In the present study, the *ACTB* gene was not a candidate for reference gene because, according to Veres-Székely et al. (2017), this gene should not be used as a reference gene when fibrosis can occur, because it generates cross-reactions of the expression of the differents actin isoforms, influencing its gene expression.

The expression analysis of genes involved with rats periodontal disease were normalized with the *GAPDH* gene, validated with stable Cq values and below 26 (Kirschneck et al., 2016). These results are in contrast to the study by Svingen et al. (2015), who did not find *GAPDH* as the most stable gene in any of the six healthy rats tissues studied, among the twelve genes tested. In brain and liver the *GAPDH* gene was the second most unstable gene among all studied and according to the data of the present research, it is possible to infer that this gene has its constant expression when evaluated in healthy tissues, which suggests that

the standardization of the use of *GAPDH* as reference gene for the species *Rattus norvegicus* could generate undue interpretations.

This research in question reveals that the stability of the *GAPDH* gene is large in the samples tested. However, there are differences in stability values in the different algorithm used and, besides that, the candidate gene reference varies according to the tissue and experimental condition imposed (Fedoseeva et al., 2014). Considering this information, it is expected that there will be differences in the stability and the level of gene expression in different researches, even if they are performed with animals of the same species.

In obese animals, when compared to healthy animals, the results corroborate with other recent studies, which have reported that *GAPDH* is widely used for the normalization of RT-qPCR reactions in an experimental model of obesity in rats (Nagao et al., 2017; Rahman et al., 2017). There are recent researches in foot lesions and muscle tissue of obese rats using *RPL32* as a reference gene without genic validation (Farnsworth et al., 2018; Lambert et al., 2018) as the reference gene. According to the results of the present study, this gene would not generate results of high reliability due to the instability in its expression at the cardiac tissue. However, each experimental situation and each tissue in question should be previously validated.

The obese and diabetic rats, when compared to healthy animals, showed *GAPDH* gene as the one with lower Cq and ΔCt, being more expressed and with less variability in these animals. Although no mention was made of *GAPDH* validation, Bortolin et al. (2017) found that, in comparison to other reference gene candidates. The *GAPDH* gene Cq did not show significant variations in rats bone tissue between the control group and the type 1 diabetes mellitus group. However, other studies (Ferreira et al., 2017; Perry et al., 2017) also use *GAPDH* for normalization of reactions with type 1 or type 2 diabetic animals without mention of the previous validation of the gene, which may sub- or overestimate the expression of target genes.

When using the geNorm algorithm in injured vocal fold tissue of rats, the *GAPDH* and *ACTB* genes were the least stable, while *SDHA* (subunit A of the succinate dehydrogenase complex) and *SPTBN1* (non-erythrocyte beta 1) were the most stable (Chang et al., 2010). In the present work, the *GAPDH* gene was not considered the most stable, by the geNorm algorithm, only in obese and diabetic animals. These findings corroborate the hypothesis that the expression of the gene varies according to the tissue in the experiment,. not with the algorithm used for normalization, and RefFinder is an instrument of high reliability.

In addition, the results of the ideal reference genes may vary among the algorithms. Expressions of eight reference genes commonly used to normalize reactions in cortical neurons of rats on the ninth and twelfth day of infection by the Borna disease virus (BDV) were evaluated. The geNorm algorithm identified the *ARBP* (attachment region biding protein) and *HPRT* (hypoxanthine guanine phosphoribosyl transferase) genes as the most stable on the ninth day of the experiment and *GAPDH* and *YWHAZ* (tryptophan 5-monoxyigenase activation protein zeta) on the twelfth day. Already the NormFinder algorithm identified *ARBP* and *ACTB* as the best for the ninth day of experiment while *ACTB* and *YWHAZ* were the best for the twelfth day. (Zhang et al., 2014). In another study with BDV, the *U87* gene was considered the most stable in geNorm, NormFinder, and BestKeeper algorithms. In comparative ΔCt the same gene was only no more stable than *MIR-101A* (microRNA 101a). However, this was considered by the final ranking as the most stable reference gene (Mao et al., 2016). With this in mind, the creation of RefFinder is justified, aiming to acquire information of greater validity.

The *POLR2A* gene has been validated as a gene suitable for muscle biopsy in humans under testosterone therapy (Petersson et al., 2014). This is consistent with the hypothesis that the best reference gene is independent of the species (Silva et al., 2015; Si et al., 2016), considering that, in the present study, the *POLR2A* gene was the second most stable for cardiac tissue of rats with metabolic alterations. Other recent studies with obese rats have used it as a reference gene (Zhou et al., 2017; Daneshpajooh et al., 2017) which, according to the present study, could compromise the validity of the study, the main failure being the non-validation of the reference gene candidates in the tissues and situation studied (renal and pancreatic cells, respectively).

To generate results of greater reliability and accuracy in gene expression, considering all reference gene candidates together is essential when several of them are used. However, the normalization of, for example, eight reference genes becomes infeasible when only two target genes need to be studied (Vandesompele et al., 2002). An ideal reference gene is one that exhibits constant expression in that experimental situation, not during the entire life of the animal (Mathur et al., 2015). This finding corroborates the study by Si et al. (2016), who found differences in the expression stability of this same gene depending on the lipid content of the diet offered to the animal, being this delayed expression when compared to the control group. Therefore, in the present research, due to obesity induction being performed with the supply of a hyperlipidic diet, the validation of the reference gene is of crucial importance in the correct interpretation of the final data.

In a recent systematic review, 1700 papers were evaluated and found that most had inadequate standardization procedures or did not possess them (Bustin et al., 2013) and some recent studies actually do not mention validation of genes (Li et al., 2014; Silva et al., 2015; and Marcioli et al., 2018). One of the possible causes of this fact is the lack of information about the importance of the validation of the reference genes in each tissue to be studied, not only in the studied species. In addition, the papers cited above are published in renowned journals, which generates high reliability in the research methodology and less fear, by the researches, at the time of perform new studies.

As regards validation of the most stable reference gene, the *CMA1* gene was used, which translates to chymase, the main enzyme responsible for the production of angiotensin II in the heart of rats (Ahmad et al., 2014). In relation to the present study, chymase was less expressed in obese animals, when the more stable reference genes were used. When less stable genes are used for normalization, it was possible to observe great variability in the expression of this gene. The ideal genes for normalization resulted in smaller standard deviations, which guarantees greater reproducibility of the results. Obesity is a metabolic change that is related to high blood pressure, thus, according to the study by Ahmad et al. (2014), it would be reasonable to find an increase in the expression of this gene in this group of animals. However, when compared to the most unstable reference genes its expression increased substantially, representing the highest value of all groups analyzed. This corroborates with other studies, which highlight the importance of the use of stable reference genes and the validation of the candidates for each research (Kirschneck et al., 2016; Li et al., 2014).

It has been observed that reducing the variability obtained by adding a high number of reference genes does not overcome some disadvantages, such as the time and the additional cost generated with such inclusions. Different genes are recommended according to each sample group and the number of reference genes used. For this paper, the use of *CMA1* gene for validation is justified, because the change in cardiac diastolic function is directly related to increased chymase expression (Wang et al., 2016).

Based on this study, it can be inferred in the present study that obese animals showed alterations in cardiac diastolic function, according to the less stable reference genes. However, when using the validated reference genes for this research, the most stable, it was demonstrated that the expression of the *CMA1* gene was lower in the obese animals when compared to the other groups of the research. This reiterates, once again, the importance of

gene validation for each research, in order to obtain results that reflect the actual experimental situation in question.

There is no universal reference gene that can be used with its best efficacy under any metabolic disturbe (obese. obese and diabetic or healthy animals), considering that genes participate in different cellular functions. In addition, modification of the tissue under study compromises the stability and validity of the same candidates for reference genes. The combination of more reference genes does not reduce the variability of gene expression, only makes the research more expensive.

This reiterates the need to analyze the optimal number of reference genes required for the normalization of reactions and to resort to a systematic gene validation that, in the present research, indicate the use of two validated stable reference genes is the most appropriate. These techniques make it possible to verify the possible reference genes in each experimental condition, aiming to increase the validity and accuracy of the research.

5. Considerações Finais

It is not possible to establish an ideal reference gene for a particular specie. The experimental situation will influence gene stability, as this changes the cellular metabolism. By establishing the optimum number of two reference genes it is possible to reduce the investment needed in the research, not losing the accuracy and reliability of the research. Thus, it was possible to show with this experiment that the most stable reference genes for RT-qPCR in the cardiac tissue of rats submitted to obesity and diabetes were *GAPDH* and *POLR2A*, respectively.

For future studies, we suggest that, when adding the RT-qPCR analysis to your research, you also make the careful selection of the best reference gene for your study, specifically, avoiding using common or random reference genes. We reiterate that each species, type of tissue and metabolic situation can generate changes in the stability of reference genes, where lies the importance of these analysis as a previous stage.

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