Int J Clin Exp Pathol 2015;8(4):3946-3954
www.ijcep.com /ISSN:1936-2625/IJCEP0006758

Original Article

Selection of reference genes for expression analyses in liver of rats with impaired glucose metabolism

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Received February 5, 2015; Accepted March 30, 2015; Epub April 1, 2015; Published April 15, 2015

Abstract: Hepatic gene expression studies are vital for identification of molecular factors involved in insulin resistance. However, the need of normalized gene expression data has led to the search of stable genes which are useful as a reference in specific experimental conditions. The aim of this study was to evaluate expression stability of potential reference genes for real-time PCR gene expression studies, in rats with insulin resistance, early programmed in intrauterine environment of maternal insulin resistance and triggered by exposure to a high sucrose and fat diet in adult life. Male rats coming from insulin resistant (F_1IR) mothers or normal (F_1N) mothers were fed a standard rodent diet from postnatal day 21 to day 56, and then divided in two groups each. One of each subgroups were fed a high sucrose and fat diet (groups F_1IR + HSFD and F_1N + HSFD respectively), the rest were fed a control diet (groups F_1IR + CD and F_1N + CD) for 14 days. Glucose metabolism related tests were later performed. After liver extraction, RNA was isolated and gene expression analyzes of seven potential reference genes (*Actb, Gapdh, Gusb, Hprt1, Ldha, Rpl13a* and *Rplp1*) were carried out. LinRegPCR software was used to analyze raw data and determinate baseline corrections, threshold lines, efficiency of PCR reactions and corrected *Cq* values. Evaluations of gene expression stabilities as well as the number of necessary genes for normalization were assessed with geNorm tool. All samples from all groups showed acceptable PCR amplification efficiencies. The most stable genes were *Rplp1*, *Ldha*, *Hprt1* and *Rpl13a* and the less stable was *Gapdh*. For all groups, just 2 to 3 of the most stable genes were necessary for optimal gene expression data normalization in rat liver. Genes encoding ribosomal proteins are the most appropriated for normalization of expression data in the presented animal model. By contrast, *Gapdh*, one of the most used genes in normalization, is not recommendable due to its high intergroup variation.

Keywords: Reference genes, gene expression, real-time PCR, geNorm, insulin resistance

Introduction

Insulin resistance occurs when a defect of insulin action on its target organs, such as skeletal muscle, liver and adipose tissue exist [1], and is one of the hallmarks of disorders like metabolic syndrome and diabetes [2]. The evidence suggests that insulin resistance is the common underlying factor beneath the metabolic syndrome and is linked to conditions such as cardiovascular disorders [3] or Alzheimer disease [4, 5], and therefore, becoming an important public health problem [6, 7].

Insulin resistance is a result of the malfunction of insulin signaling recognition at cellular level, especially in tissues like skeletal muscle, adipose tissue and liver. The contribution of liver to the onset of an insulin resistance state is recognized mainly due to the important role of this organ in energetic homeostasis, involving lipid and carbohydrate metabolism. Liver seems to be one of the first organs in which the insulin gradually losses its action, beside skeletal muscle, towards to a frank insulin resistance state [8]. Hepatic fat accumulation is one of the pivotal events in the establishment of hepatic insulin resistance, further decreasing its insulin response, creating a vicious circle that finally converges in liver steatosis and Nonalcoholic fatty liver disease.

Environmental and genetic factors have been related to insulin resistance and its complica-

tions. It is well known that high fat or sucrose diets (or a combination of both) lead to development of impaired glucose tolerance and insulin resistance [9, 10]. However, the identification of genetic variants that accurately predict the onset of insulin resistance remains elusive [2]. On the other hand, the intrauterine environment seems to play a key role in the risk of occurrence of adult metabolic disorders, including obesity, insulin resistance and type 2 diabetes [11, 12]. However, few studies have established a link between gene expression profile and the onset of metabolic abnormalities in offspring exposed to high sucrose and fat diets (HSFDs), coming from mothers with insulin resistance. The latter could be due to the difficulty of analyzing non-genetic heritable characteristics in early stages of development and the impact of intrauterine environment in the potential risk of establishment of late onset metabolic diseases in humans. Animal models are very helpful tools to investigate these issues. Gene expression analyzes in these models can provide important information about molecular pathways involved in the early programming of metabolic disorders. Moreover, real time PCR is a powerful, fast and accurate tool for gene expression analyses. However, difficulties inherent to this method exist, being the right choice of adequate reference genes for normalization one of the most challenging. Consequently, the need arises to choose an optimal set of reference genes for each experimental model, and tools emerged to perform this choice in a statistically founded, standardized form.

In the present study, we used the bioinformatic tool geNorm to analyze the stability of seven potential reference genes in a model of rat with impaired glucose metabolism, in order to establish a set of optimal reference genes useful for gene expression studies in this model.

Materials and methods

Experimental design

All experiments followed the Guide Principles for the Care and Use of Animals based on the Helsinki Declaration. In order to generate an animal model that allowed us to study the impact of maternal insulin resistance and intrauterine factors in the early programming of late onset impairments on glucose metabolism, the study was designed in two phases.

In the first phase, a female insulin resistant rat model (F_0 rats) was developed. For this purpose, female Wistar rats (10 ± 1 weeks of age) were fed a high sucrose and fat diet (HSFD; 40% carbohydrate 40% fat, 20% protein) or a controlled diet (CD; 64% carbohydrate 15% fat, 21% protein) for eight weeks. Afterwards, all animals were submitted to measurements of fasting glucose and insulin levels and Glucose tolerance test (GTT). Later, the normal (CD treated) and the insulin resistant (HSFD treated) rats were mated with healthy males, using a harem system. Pregnant animals were isolated and fed a standard rodent diet until the end of the gestational period.

The second phase investigation involved the study of the male offspring (filial 1, F_1) of normal (F_1N) and insulin resistant (F_1IR) animals. At postnatal day 3, F_1 animals were sexed, and at the end of the lactation period, male F_1 rats were housed in groups of 4 to 5 individuals. From week 3 to 8, male F_1 animals were fed a standard rodent diet and then each group was divided into two subgroups. One group from F_1IR and one from F_1N were fed a HSFD (groups F_1IR + HSFD and F_1N + HSFD respectively) and the rest were fed a control diet (groups F_1IR + CD and F_1N + CD) for 14 days. Afterwards, GTT, pyruvate (PTT) and insulin tolerance tests (ITT) were performed, in addition to body weight and basal insulin levels measurements.

Tissue collection and RNA isolation

After 6 hours fasting, rats were euthanized, the livers were isolated, frozen in liquid nitrogen and stored at -80°C until processing. RNA was isolated from ~50 mg of tissue, using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and a mechanical tissue disruptor for homogenization, followed by purification with RNeasy mini kit (Qiagen, Hilden, Germany). On column treatment with DNase I (Qiagen) was also performed, previous to quantification using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated in denaturing TAE agarose gels (28S/18S rRNA ratio ≥ 2) and the purity was verified using OD260/OD280 nm absorption ratio > 1.8 and OD260/OD230 nm absorption ratio > 2.0.

Reverse transcription and Real-time PCR

Reverse transcriptions were performed with SuperScript III Reverse Transcriptase (Life

Table 1. Primer sequences used for gene quantification in Real-time PCR experiments

Gene	Gene Bank Access N°	Primer	Sequence	Product size (bp)
Beta actin (*Actb*)	NM_031144.3	Actb-F	GACCCAGATCATGTTTGAGACC	77
		Actb-R	AGGCATACAGGGACAACACA	
Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)	NM_017008.4	Gapdh-F	CTCTCTGCTCCTCCCTGTTC	101
		Gapdh-R	GCCAAATCCGTTCACACCG	
Glucoronidase beta (*Gusb*)	NM_017015.2	Gusb-F	GACGTTGGGCTGGTGAACTA	97
		Gusb-R	GGGCCACAATTTTGCCATCC	
hypoxanthine-guanine phosphoribosyltransferase (*Hprt*)	NM_012583.2	Hprt-F	TAGCACCTCCTCCGCCAG	96
		Hprt-R	CACTAATCACGACGCTGGGA	
Lactate dehydrogenase (*Ldha*)	NM_017025.1	Ldha-F	GATCTCGCGCACGCTACTG	73
		Ldha-R	TTTGAATCTTTTGGGACCGCTT	
Ribosomal protein L13A(*Rpl13a*)	NM_173340.2	Rpl13a-F	GCAAAGATCCATTACCGGAAG	139
		Rpl13a-R	ACAGTCTTTATTGGGTTCACAC	
Ribosomal protein, large, P1 (*Rplp1*)	NM_001007604.2	*Rplp1-F*	TAAGGCCGCGTTGAGGTG	150
		Rplp1-R	GATCTTATCCTCCGTGACCGT	

Table 2. Characteristics of F_0 female rats at the end of administration with control or high sucrose and fat diet

	Control (N = 5)	HSFD (N = 10)	*P* Value
Weight (g)	274.4 ± 4.2	268.1 ± 5.2	*NS*
Length (cm)	41.32 ± 0.93	42.02 ± 0.72	*NS*
PGL (mM)	7.25 ± 0.12	7.43 ± 0.20	*NS*
Plasmatic Insulin (ng/mL)	1.05 ± 0.22	1.86 ± 0.21	0.0007
AUC of glycemias during GTT	1216 ± 18.07	1579 ± 41.84	< 0.001

HSFD: High Sucrose and Fat Diet; PGL: Plasmatic Glucose; AUC: Area under the curve; *NS*: Not statistically significant. Values presented as Mean ± S.E.M.

Technologies, Carlsbad, CA, USA) and 1 µg of purified RNA. In order to improve the reverse transcription, a combination of olygo-dTs and random primers were used. All qPCR reactions were run in triplicate. PCR reaction mix (20 µL) were prepared with 200 ng of cDNA, 100 mM of specific forward and reverse primers and 1x Maxima SYBR Green PCR Mastermix (Fermentas, Burlington, Canada). Primers were designed using NCBI's Primer-BLAST online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers used are listed in **Table 1**. The cycling was performed in a Rotor-Gene Q real time PCR system, with an initial polymerase activation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. In order to check the quality of primers, a dissociation curve was performed at the end of the run.

Gene expression analyses

Seven potential reference genes (*Actb, Gapdh, Gusb, Hprt1, Ldha, Rpl13a* and *Rplp1*) were analyzed. qPCR raw data was exported into an Excel datasheet and analyzed using LinRegPCR software [13], which evaluate efficiency and linearity range, in addition to determine Thresholds and *Cq* for each gene and sample. Efficiency value over 1.7 was considered as acceptable. The *Cq* normalized data values were transformed to Raw Quantities of transcript (RQ) through Delta-*Cq* method ($RQ = E^{-(\Delta Cq)}$), considering amplification efficiency for each primer pair [14, 15]. GeNorm software was used to determinate the internal control gene-stability measures (*M*) and the Pair-wise variation (*V*) between the two sequential normalization factors (NF_n and NF_{n+1}), in order to evaluate the optimum number of reference genes for normalization. A *V* value cut-off of 1.5 is recommended and therefore, the inclusion of an additional reference gene under the previously mentioned value is unnecessary [16]. These analysis were performed separately in four sample groups (F_1N + CD, F_1IR + CD, F_1N + HSFD and F_1IR + HSFD) and together (all groups).

Statistical analysis

The data related to phenotypic and metabolic characteristics of animals was presented as Mean ± S.E.M. Multiple groups comparisons were analyzed with ANOVA and Tukey's post-test if the data had a normal distribution. On the contrary if it did not show a Gaussian distribution Kruskal-Wallis test with Dunns's test were performed. *P* value < 0.05 was considered statistically significant.

Table 3. Characteristics of F_1 male rats after 14 days administration of control or high sucrose and fat diet

Parameters	Groups			
	F_1N + CD	F_1N+ HFSD	F_1IR + CD	F_1IR + HFSD
Body Weight (g)	295.4 ± 7.5	307.2 ± 5.8	345.1 ± 9.3[a]	348.2 ± 5.9[a]
Body Length (cm)	38.1 ± 5.3	36.9 ± 4.9	36.7 ± 6.2	40.2 ± 2.9
PGL (mM)	5.958 ± 0.24	6.084 ± 0.61	6.584 ± 0.22	6.779 ± 0.31
Plasmatic Insulin (ng/mL)	0.63 ± 0.09	0.81 ± 0.12	0.80 ± 0.15	1.14 ± 0.27[a]
AUC of glycemias during GTT	39.8 ± 2.3	50.1 ± 4.4	47.1 ± 1.7	68.4 ± 4.2[a,b,c]
AUC of plasmatic insulin during GTT	73.8 ± 7.1	108.4 ± 5.5	111.3 ± 6.4 [a]	161.7 ± 10.2[a,b,c]
AUC of glycemias during ITT	19.7 ± 4.3	19.3 ± 2.3	23.1 ± 2.9	21.7 ± 2.4
AUC of glycemias during PTT	55.7 ± 7.3	68.6 ± 6.5	74.3 ± 9.4	90.6 ± 14.0[a]
WAT weight (g)	7.8 ± 3.5	10.1 ± 3.7	11.1 ± 4.3	14.6 ± 4.1[a]
Hepatic Lipids content ($mg{\cdot}mL^{-1}$/g of tissue)	12.2 ± 4.1	17.8 ± 4.7	21.9 ± 5.5	64.6 ± 9.1[a,b,c]
Hepatic Glycogen ($mg{\cdot}mL^{-1}$/g of tissue)	38.3 ± 2.4	32.1 ± 3.3	31.7 ± 2.1	24.2 ± 1.9[a]

PGL: Plasmatic Glucose; AUC: Area under the curve; GTT: Glucose tolerance test; ITT: Insulin tolerance test; PTT: Pyruvate tolerance test; WAT: White adipose tissue. Values presented as Mean ± S.E.M. [a]: Statistically significant vs. F_1N + CD; [b]: Statistically significant vs. F_1N + HSFD; [c]: Statistically significant vs. F_1IR + CD.

Table 4. PCR efficiencies and correlations of analyzed genes

Gene	Efficiency	Correlations (R^2)
Beta actin (*Actb*)	1.875	0.99995
Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)	1.739	0.99989
Glucoronidase beta (*Gusb*)	1,840	0.99975
Hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt-1*)	1.898	0.99996
Lactate dehydrogenase (*Ldha*)	1.937	0.99998
Ribosomal protein L13 A (*Rpl13a*)	1.878	0.99996
Ribosomal protein, large, P1 (*Rplp1*)	1.922	0.99998

Results

Phenotypic characteristics of animals

Characteristics of F_0 female rats at the end of HFSD (Insulin resistant) or Control Diet (Normal) supplementation are shown in **Table 2**. Animals did not show significant differences in body weight, plasmatic glucose nor cephalocaudal length, between HFSD and Control Diet groups at the end of dietary manipulation. However, HFSD treated animals showed a significant increment in basal insulin levels in addition to increased area under the curves of glycemia during GTT.

Respecting to F_1 animals, F_1IR pups showed significant increment in body weight at postnatal day 3 (16.07 ± 0.61 g vs. 20.53 ± 0.59 g, $P < 0.001$) but not in plasmatic glucose, when compared with F_1N pups. These trends in body weight and plasmatic glucose remained the same at postnatal day 21 and 56. The characteristics of F_1 male rats at day 70, after 14 days of dietary manipulation, are shown in **Table 3**. F_1IR + DASG rats showed impaired glucose tolerance, basal hyperinsulinemia and elevated insulin levels during GTT, in addition to increased white fat depots, and hepatic lipid content when compared with the others groups.

Real time PCR reaction quality control and data normalization

All samples yielded significant amounts of RNA. 260/280 nm and 260/230 nm indexes were higher to 1.8 and 2.0 respectively, indicating acceptable purity of isolated RNA.

Real-time PCR data correction was performed using LinRegPCR program. In all analyzed samples, the efficiency of reactions was near to 2 and R^2 values were consistent with optimal correlations. The efficiencies values are shown in **Table 4**. Once baselines were corrected, the window of linearity and the threshold line were

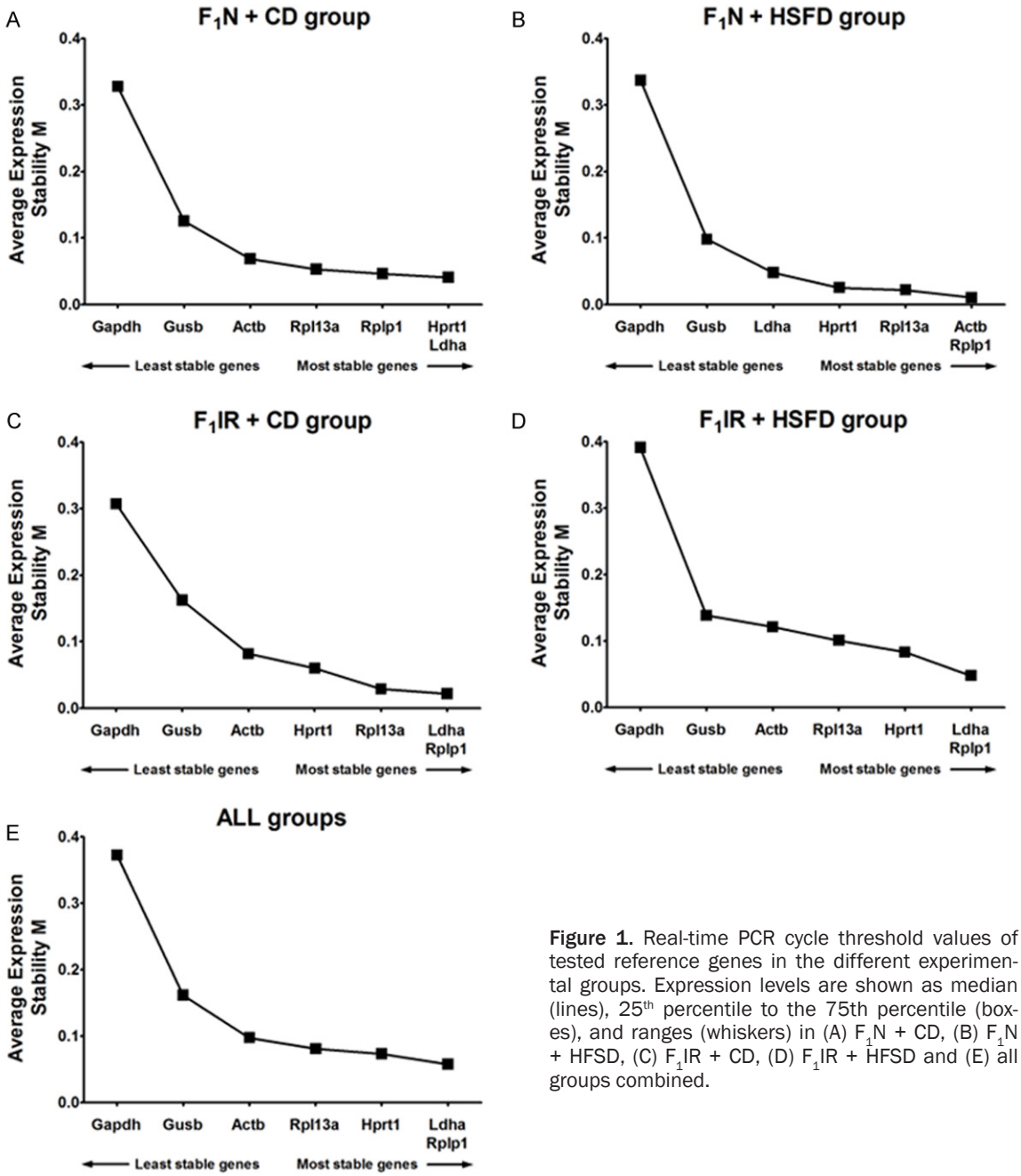

Figure 1. Real-time PCR cycle threshold values of tested reference genes in the different experimental groups. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers) in (A) F_1N + CD, (B) F_1N + HFSD, (C) F_1IR + CD, (D) F_1IR + HFSD and (E) all groups combined.

calculated, resulting in a value of 5.389 (expressed as log of fluorescence).

Real time PCR data analysis

After data normalization, the quantification cycle (*Cq*) was calculated for all PCR reactions, considering the corrected efficiencies for each gene. Analyzed reference genes showed variation in *Cq* values ranging from 21.19 to 25.91 for F_1N + CD group, 20.13 to 25.90 for F_1N + HFSD group, 19.33 to 25.64 for F_1IR + CD group, and 19.29 to 25.50 for F_1IR + HFSD group (**Figure 1**).

Expression stability of reference genes

Expression stability of the seven potential reference genes was analyzed using geNorm software. The studied genes showed expression stability measures (M values) between 0.252 (*Rplp1*) and 0.899 (*Gapdh*) when analyzed together. Reference genes stability analysis showed that the less variable gene was *Rplp1*,

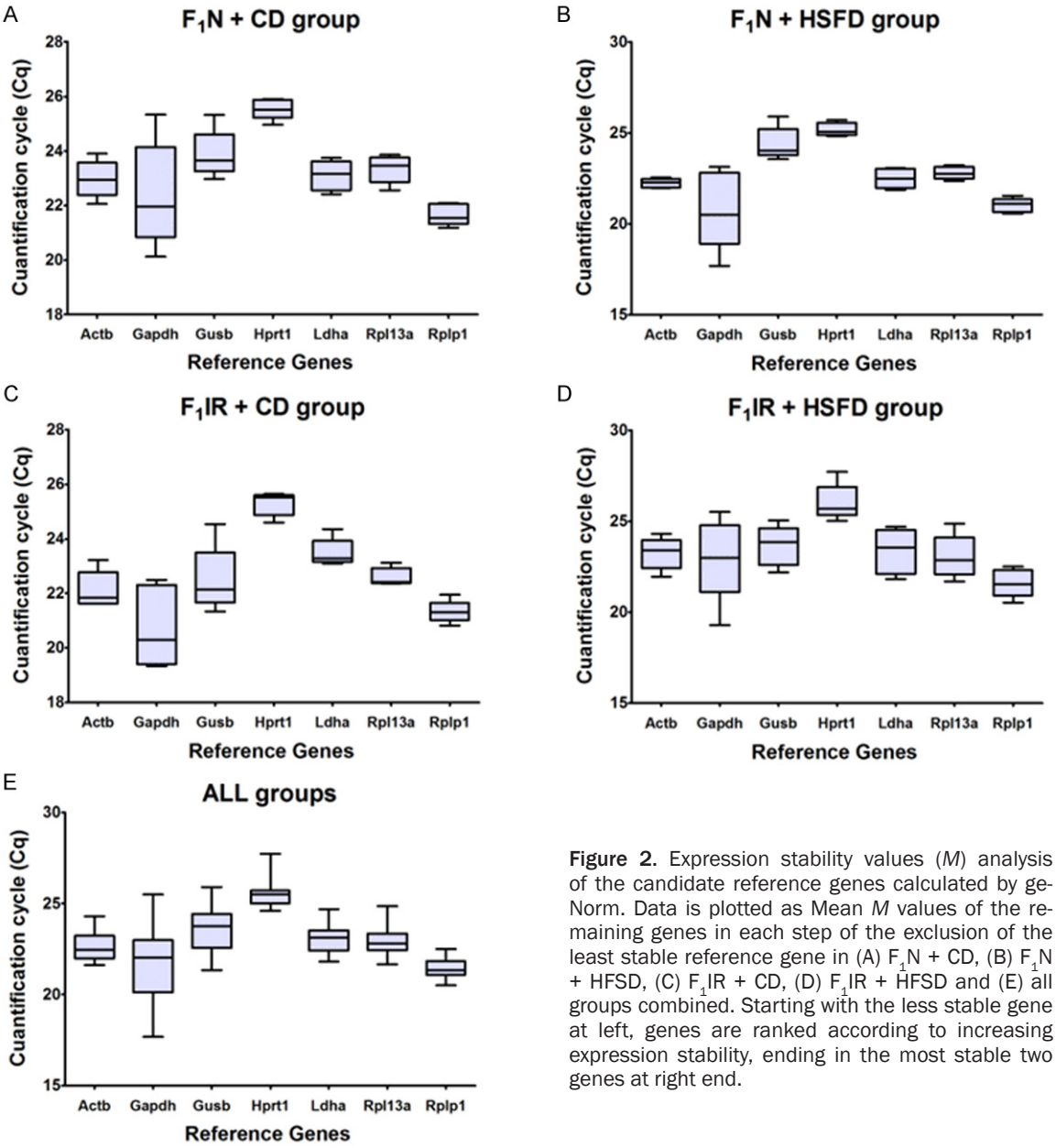

Figure 2. Expression stability values (*M*) analysis of the candidate reference genes calculated by geNorm. Data is plotted as Mean *M* values of the remaining genes in each step of the exclusion of the least stable reference gene in (A) F_1N + CD, (B) F_1N + HFSD, (C) F_1IR + CD, (D) F_1IR + HFSD and (E) all groups combined. Starting with the less stable gene at left, genes are ranked according to increasing expression stability, ending in the most stable two genes at right end.

which was part of the lesser variable pair in all data groups analyzed, with exception of F_1N + NC. In addition to *Rplp1*, *Ldha*, *Hprt1* and *Rpl13a* were the most stable genes among all analyzed samples. *Gapdh* showed the highest M value, demonstrating to be the less stable gene in all analyzed groups. Expression stability of candidate reference genes are shown in **Figure 2**.

As presented in **Figure 3**, the pairwise variations V2/3, V3/4, V4/5, V5/6 and V6/7 were all lower than the limit value of 0.15, indicating that the addition of a third reference gene for normalization would be not required, and could even diminish the accuracy of normalization. Since all pairwise variations were below 1.5, even when the data was analyzed for each experimental group or in a single set grouping all data, the above observation is still valid.

Discussion

The establishment of well characterized animal models is vital to fully understand the condition that the model represents. In metabolic alterations, this issue is a challenge, because of the great number of tissues and organs involved.

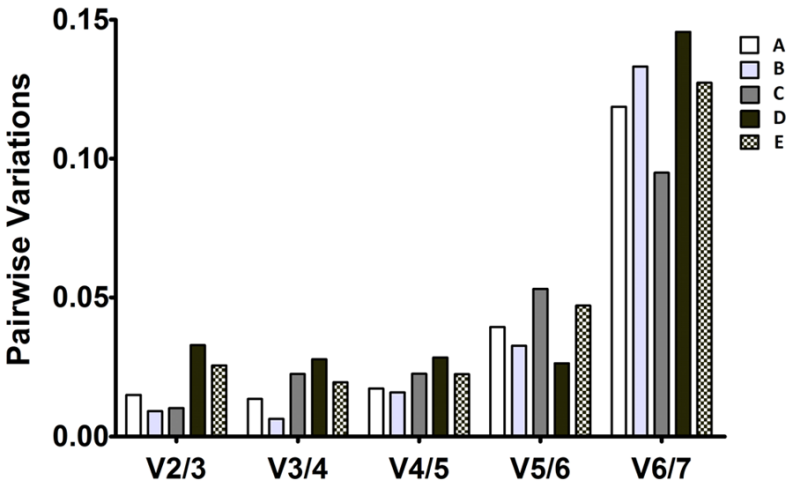

Figure 3. Expression stability values (*M*) analysis of the candidate reference genes calculated by geNorm. Data are plot in (A) F_1N + CD, (B) F_1N + HFSD, (C) F_1IR + CD, (D) F_1IR + HFSD and (E) all groups combined.

Furthermore, it becomes highly difficult if non genetic heritable risk factors are willing to study. Due to this, an appropriate characterization is not only optional, but necessary, when animal models are used. In our model, the absence of genetic alterations is one of the principal advantages, which makes it useful for the study of heritable epigenetic factors which determine the early programming of late onset insulin resistance. Another advantage is the absence of drugs utilization to cause deleterious effects on glucose metabolism, such as streptozotocin and alloxan, which can have undesirable side effects that can lead to confounding results, when subsequent generations of animals are studied. However, due to scanty validated information available about gene expression patterns in different tissues of animals with disorders in glucose metabolism derived from maternal insulin resistance and short exposure to high caloric diets, molecular characterization is needed to help clarify the molecular basis of metabolic alterations observed in animals fed with high sucrose and fat diet which also come from insulin resistant mothers. Considering the latter, liver appears to be one of the main actors in these alterations, and therefore, was chosen as a pilot tissue for gene expression analysis.

Gene expression studies trough real-time PCR analysis are the most common strategy applied to identify relevant changes in gene regulation processes that may be involved in biological and pathological processes. This strategy offers rapid and consistent results. However, it is necessary to validate in detail the parameters involved in this technique, as a previous stage to experiment running. Accordingly, one of the critical points in validation is to select appropriate reference genes for data normalization in gene expression studies, in order to guarantee the correct analysis of observations and the quality of results.

The use of reference genes as internal control is commonly accepted as the most appropriate strategy in qPCR data normalization [17], however, their utility must be experimentally validated for particular experimental designs [18]. Utilization of a single reference gene in normalization is not recommended. Therefore, which and how many genes are needed for this purpose must be experimentally determined [16, 19, 20]. An inadequate reference genes selection can result in altered results, particularly when exist small transcription rate variations between sample groups [21, 22].

Appropriated reference genes must ideally show consistent expression levels in all samples and experimental conditions [21]. However, has been demonstrated that under different experimental conditions, the expression among genes could differ.

In our study, we evaluated seven potential reference genes in liver of insulin resistant male rats. *Rplp1* and *Ldha* showed the greatest stability in their expression, followed by *Rpl13a* and *Hprt1*. However, *Gapdh* and *Gusb* were the least stable. It seems to be a usual practice using just one gene as normalizer of expression data. In addition, many works in real-time PCR still choose “housekeeping” genes like *Actb* and *Gapdh*, even when it has been reported that these common reference genes are not stably expressed in various experimental conditions [23, 24]. In fact, *Gapdh* has been reported as a gene that shows lower expression stability [25-27].

After estimation of M value, geNorm calculates the number of minimum necessary genes for a

proper normalization. In our study, very lower V values were found with almost all studied genes, needing just the three or even the two most stables. In fact, when all samples were analyzed as a single dataset, the use of just the three most stable genes (*Ldha*, *Rplp1*, *Rpl13a*) was enough for gene expression data normalization. Our results are consistent with previously published works, showing that genes coding for ribosomal protein such as *Rpl32*, *Rpl17*, *Rlpl1* and *Rpl13a*, have the best performances as reference genes, even in many different tissues and experimental conditions [25, 27-29].

In summary, an appropriate selection of reference genes is a key step in characterization of an animal model. In fact, in a model of rat with insulin resistance programmed by intrauterine environmental factors and triggered for a short exposure to a high sucrose and fat diet, some ribosomal proteins and a gene coding for a constitutive enzyme (*Ldha*) were the best for expression data normalization. These findings are useful tools for subsequent gene expression studies in the liver of rats belonging to this animal model, ensuring in part the robustness of future analysis. However, this work is just a first step. Information about appropriated reference genes for other tissues that are important in the insulin resistance pathological process, like skeletal muscle and adipose tissue, must be also performed, in order to optimize genes expression analyses in these tissues, helping to a better understanding of molecular mechanism of metabolic alteration described in our animal model.

Acknowledgements

This work was supported by CONICYT-Chile, FAPESP-Brazil, and Guggenheim Foundation.

Disclosure of conflict of interest

None.

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