MARINA MARTINS DE OLIVEIRA

VALIDAÇÃO DE GENES DE REFERÊNCIA PARA RT-qPCR EM TECIDO CARDÍACO DE RATOS SUBMETIDOS À OBESIDADE E AO DIABETES

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Ciências Veterinárias, para obtenção do título de Mestre.

Orientadores

Prof. Dr. Raimundo Vicente de Sousa (*in memoriam*)

Prof. Dra. Ana Paula Peconick

Coorientador(a)

Kalynka Gabriella do Livramento

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APROVADA em 16 de julho de 2018.

Dr. Elaine Maria Seles Dorneles	Universidade Federal de Lavras
Dr. Fabrício Luciani Valente	Universidade Federal de Viçosa
Dr. Rafael Neodini Remedio	Universidade Federal de Lavras

Orientadores

Prof. Dr. Raimundo Vicente de Sousa (*in memoriam*)

Prof. Dra. Ana Paula Peconick

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“A sua partida não ofuscará seu brilho”. Ao Prof. Dr. Raimundo Vicente de Sousa.

DEDICO

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RESUMO

O presente trabalho tem 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 aos 42 dias de vida, sendo a eutanásia realizada no 70º dia. Os corações dos animais foram coletados e o ápice cardíaco foi destinado às técnicas moleculares para extração do RNA, realizada com TRIzol®. Os candidatos a genes de referência foram *GAPDH*, *POLR2A*, *RPL32* e *RPL4*. A técnica da RT-qPCR foi feita em termociclador, sendo a eficiência dos iniciadores encontrada pelo *software LinReg* e a estabilidade da expressão dos genes de referência nas amostras analisada pelo algoritmo *RefFinder*. O gene alvo utilizado para verificar a diferença da expressão gênica dos candidatos a genes de referência foi o *CMA1*. Os resultados sugerem que os animais obesos apresentaram uma diminuição da expressão do gene *CMA1* quando comparado com os dois genes de referência mais estáveis. O contrário ocorre quando o mesmo é comparado com os dois genes de referência menos estáveis. Não existe um gene de referência universal para todas as situações, o que requer uma validação sistemática para cada uma. A utilização de genes de referência não validados pode comprometer a expressão dos genes alvo, o que impediria o reflexo da situação real. Os genes *GAPDH* e *POLR2A* são os melhores para normalizar as reações com as amostras nas condições do presente experimento.

Palavras-chave: Normalização, RT-qPCR, *RefFinder*, *GAPDH*, *POLR2A*

ABSTRACT

The present work aims to identify and validate reference genes for RT-qPCR in cardiac tissue of rats of the *Rattus norvegicus albinus* species, submitted to obesity associated or not to type 2 diabetes mellitus. For this, the metabolic changes were induced at 42 days of life, with euthanasia being performed on the 70th day. The hearts of the animals were collected and the cardiac apex was assigned to the molecular techniques for RNA extraction, performed with TRIzol®. Candidates for reference genes were *GAPDH*, *POLR2A*, *RPL32* and *RPL4*. The RT-qPCR technique was done in thermocycler, with the efficiency of the primers found by the *LinReg* software and the stability of the expression of the reference genes in the samples analyzed by the *RefFinder* algorithm. The target gene used to verify the difference in gene expression of candidates for reference genes was *CMA1*. The results suggest that 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. There is no universal reference gene for all situations, which requires systematic validation for each. The use of unvalidated reference genes may compromise the expression of the target genes, which would prevent the reflection of the actual situation. The *GAPDH* and *POLR2A* genes are best at normalizing the reactions with the samples under the conditions of the present experiment.

Keywords: Normalization, RT-qPCR, RefFinder, *GAPDH, POLR2A*

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1 INTRODUÇÃO

A rapidez da técnica da transcriptase reversa da reação em cadeia da polimerase em tempo real quantitativa (RT-qPCR), associada à sua sensibilidade e alto rendimento, possibilita a avaliação e quantificação da expressão gênica em diversos tecidos animais, dentre eles o cardíaco. A técnica está sendo progressivamente mais utilizada, o que reflete no aumento do número de publicações de trabalhos com técnicas de RT-qPCR (BRATTELID et al., 2010). Entretanto, a obtenção de resultados confiáveis depende da utilização de genes de referência adequados, que serão utilizados para normalizar a expressão gênica entre as amostras. Vários estudos relatam que não existe um gene de referência padrão para todos os tecidos e condições experimentais (CHEN et al., 2017; CHANG, et al., 2016; FREITAS et al., 2017). Dessa forma, a identificação dos melhores genes de referência para um experimento em determinadas condições é essencial para a aplicação dos métodos em RT-qPCR (CHEN, 2017).

Vários fatores podem interferir na normalização de uma reação de RT-qPCR, como a quantidade de material inicial e as diferenças na transcrição gênica entre tecidos e células. Para se obter uma expressão basal a ser comparada com a expressão dos genes alvo é realizado o emprego dos genes de referência, como o *GAPDH* (gliceraldeído-6-fosfato desidrogenase) ou o *ACTB* (β-actina) (NISHIMURA et al., 2006). Entretanto, a escolha do gene de referência ideal vai depender da espécie, do tipo de tecido e das condições experimentais as quais os animais foram submetidos. Para isso, é feita a validação dos candidatos a gene de referência (DO LIVRAMENTO et al., 2018). A utilização de genes de referência, anteriormente considerados preconizados para dada espécie, pode prejudicar os resultados por não apresentar uma expressão constante em determinado tecido ou condição experimental, como seria esperado (MATHUR et al., 2015).

A obesidade e o diabetes são doenças multifatoriais que podem acarretar prejuízos para outros sistemas orgânicos, como o renal e o cardíaco (CEFALU et al., 2015). Ademais, a hiperglicemia, resultante da deficiência da produção de insulina ou da resistência à mesma, gera a formação de espécies reativas de oxigênio (EROs). De forma crônica, a exposição às EROs pode aumentar a apoptose de cardiomióticos e o acúmulo de tecido fibroso no coração (SHUCKLA et al., 2017). Sendo assim, esse trabalho visa avaliar o potencial normalizador de 4 genes de referência para o tecido cardíaco de ratos obesos e diabéticos.

2 REFERENCIAL TEÓRICO

2.1 A técnica da reação em cadeia da polimerase quantitativa por transcrição reversa em tempo real (RT-qPCR)

Enquanto a PCR convencional visa multiplicar a sequência do DNA alvo a concentrações que possam ser detectadas, a PCR em tempo real monitora o progresso da amplificação pelo uso de moléculas fluorescentes (SAMBROOK e RUSSEL, 2002). Uma das maiores limitações da técnica de RT-qPCR é a instabilidade e a curta meia vida da molécula de RNA, além de saber o exato momento no qual aquele RNA que se quer avaliar foi transcrito. O ponto crucial da quantificação em tempo real do RNA é a extração, a qual deve manter a integridade e qualidade do mesmo. Ademais, é importante que não haja contaminação (DNA, proteínas, lipídios) no material extraído e que o cDNA produzido reflita com precisão a concentração de RNA inicial. A técnica de RT-qPCR pode ser utilizada para avaliar a expressão gênica, detecção de vírus RNA e pequenos RNA's (SMITH e OSBORN, 2008).

A técnica de detecção de fluorescência pela RT-qPCR é uma das mais utilizadas nos dias atuais, nas mais diversas espécies e tipos de tecido, o que reflete no maior número de trabalhos publicados com foco nessa técnica. A técnica consiste basicamente em 3 etapas: conversão do RNA mensageiro (RNAm) extraído da amostra em DNA complementar (cDNA); amplificação desse DNA complementar pela reação de PCR convencional; e quantificação da amplificação desse produto na PCR em tempo real (NOLAN et al., 2006). Para a validação dos resultados de RT-qPCR alguns fatores importantes devem ser considerados, como a pureza do RNA extraído, com base na quantificação em espectrofotômetro e resultados da eletroforese em gel de agarose; utilização de genes de referência adequados para cada experimento; e o teste da eficiência de amplificação de cada gene (CHANG et al., 2016).

Existem alguns problemas que envolvem a técnica de RT-qPCR, como a inconsistência de métodos de extração de RNA total, quantidade de RNA por reação e variação na fabricação de kits de transcrição reversa. Além disso, existem diversos métodos de detecção para RT-qPCR, como sistemas baseados em corantes ou sensores, o que amplia o espectro de aplicações (DUBOIS et al., 2013). Para obter mensurações confiáveis o pesquisador deve seguir o passo a passo de diversas técnicas e usar a aparelhagem adequada, selecionando abordagens estatísticas relevantes para cada análise de dados. Ademais, os detalhes dos experimentos devem ser descritos de forma clara, para que a pesquisa possa ser replicada com a mesma qualidade (BUSTIN et al., 2013).

Sem a transparência da otimização, validação e controle de qualidade dos procedimentos é impossível para o leitor distinguir um resultado válido de uma variação ou um erro de técnica. A publicação deve explicitar como o RNA foi extraído, como o cDNA foi confeccionado, como os iniciadores foram selecionados e como os genes de referência foram validados. Esses dados tendem a ser negligenciados pelos pesquisadores, sendo um gene de referência incluído em determinada pesquisa devido à sua utilização por outro experimento anterior. Entretanto, é sabido que um gene de referência não normaliza as reações de todos os tecidos de uma mesma espécie animal, sendo necessária a validação em todos as amostras a serem estudados. A depender da condição experimental a expressão gênica pode variar, resultando na não normalização da reação por um gene, que deveria se expressar constantemente (BUSTIN et al., 2013).

A quantificação gênica é normalmente acompanhada da normalização pelo gene de referência, constantemente expressos no organismo (CHEN, 2017). Porém, recentemente tem surgido o conceito de que o gene de referência não deve, necessariamente, ser constantemente expresso durante toda a vida do animal e sim no momento em questão, que interessa para o estudo experimental. Dessa forma é possível inferir que um gene expresso constantemente em determinada situação pode não ser em outra, demonstrando a importância da validação dos candidatos a genes de referência antes do início das análises (MATHUR et al., 2015).

A interpretação dos resultados de uma RT-qPCR é feita no ponto onde a fluorescência cruza a linha do *Cycle Threshold* (*Ct*). O valor de *Ct* é inversamente proporcional à concentração de ácidos nucleicos da reação: quanto maior o *Ct*, menor a concentração do cDNA alvo. A reação de RT-qPCR gera um gráfico que possui uma linha base. No momento em que a intensidade da fluorescência emitida pela amostra cruza a linha base é marcado o ciclo de expressão do gene correspondente ao par de iniciadores que está contido na reação. As amostras que emitirem a fluorescência primeiro são as que possuem maior concentração do cDNA em questão. Na validação dos genes que normalizarão a reação, os candidatos a gene de referência devem se expressar de forma constante, ou seja, o número de ciclos necessários para que a fluorescência cruze a linha base deve ser próximo entre as amostras em questão, independentemente de sua variação (SAMBROOK E RUSSEL, 2002).

É possível estimar o número inicial de cópias de uma amostra de DNA na técnica de RT-qPCR pelo ligante *SYBR Green*, que é mais economicamente viável do que a sonda *TaqMan*, sendo essa de maior especificidade. Entretanto, o sinal da fluorescência precisa ser identificado como proveniente do DNA alvo, o que é feito pela obtenção da curva de *melting* (AGRIMONTI et al., 2018). A curva de *melting*, ou curva de dissociação, é formada quando

metade do DNA da amostra está em fita dupla e metade encontra-se desnaturado. O aparelho revela a amplificação de produtos semelhantes quando a temperatura de fusão dos *amplicons* é também semelhante (GANOPOULOS et al., 2013), sendo utilizada para verificar a variabilidade de expressão de cada candidato a gene de referência. Quando a curva de *melting* produz um pico único significa que cada par de primer amplificou em um único produto de PCR, o que é desejável (LEE et al., 2009; WANG et al., 2013). No caso de haver dois ou mais picos na curva de *melting* é indicativo da amplificação de um subproduto inespecífico, indesejável na reação. A amplificação em pico único na curva de *melting* comprova a especificidade da amplificação (WEYRICH et al., 2010).

2.2 Genes de referência e sua validação

Os genes de referência são conhecidos por serem constantemente expressos no organismo do animal, pois são essenciais para a vida (KOZERA & RAPACZ, 2013). Um único gene de referência pode não normalizar uma reação de RT-qPCR e a estabilidade dos genes varia de acordo com a espécie e o tipo de tecido utilizado, além das condições experimentais. Isso afeta a interpretação dos resultados da técnica (KIRSCHNECK et al., 2016). A expressão gênica é modificada de acordo com a exposição do animal a diferentes estímulos, contribuindo para a formação do fenótipo dos animais. Para que essas mudanças sejam mensuradas com precisão é necessário possuir um conjunto de genes com expressão relativamente constante sob as condições em questão, sendo esses os chamados genes de referência (IYER et al., 2017).

Os genes utilizados como referência são chamados de genes *housekeepings*, pois apresentam um perfil de expressão estável. Isso ocorre porque os genes de referência são escolhidos com base em sua expressão constante durante toda a vida do animal, sendo essencial para ela. Entretanto, estudos comprovam que não há um gene de referência universal, pois a estabilidade da expressão gênica muda a depender da espécie, tipo de tecido estudado e das condições experimentais em questão (CHEN, 2017; FREITAS et al., 2017; DO LIVRAMENTO et al., 2018). Sua expressão é alterada nos casos de estresse oxidativo, inflamação, apoptose, proliferação celular. A análise da expressão gênica pode diferir a depender da escolha dos genes de referência (SHUCKLA et al., 2017).

Estudos como o de Li e colaboradores (2014) provaram a importância de realizar a análise de validação dos candidatos a genes de referência em cada experimento, considerando a variação da expressão gênica em diferentes tecidos e condições experimentais. A técnica de

RT-qPCR é um método propenso a erros devido à manipulação de amostra, qualidade do material coletado, extração do RNA e qualidade da extração. Dessa forma, a normalização da reação é realizada com objetivo de aumentar a confiabilidade da análise e evitar interferências no resultado. Porém, o gene selecionado deve ser estável e não afetado pelas condições experimentais (KOSINOVÁ et al., 2016).

A validação do gene de referência é obtida pelas reações de RT-qPCR nas amostras biológicas com os candidatos a genes de referência, sendo selecionados aqueles que possuem a menor variação e a maior estabilidade entre as amostras biológicas estudadas (DUBOIS et al., 2013). As abordagens matemáticas mais utilizadas para mensurar essa variação são o algoritmo *NormFinder* (ANDERSEN et al., 2004), o *BestKeeper* (PFAFFL et al., 2010), o *geNorm* (VANDESOMPELE et al., 2002) e o Δ*Ct* comparativo (SILVER et al., 2006).

O *geNorm* baseia-se no conceito de que a expressão de dois genes de referência ideais permanece constante em todas as amostras, independentemente da condição experimental. Dessa forma, se essa relação varia, em um ou mais genes, ele é expresso de forma instável. O valor de estabilidade é o valor de M, definido como a variação média da expressão dos candidatos a genes de referência. A estabilidade do gene aumenta à medida que o valor de M cai (VANDESOMPELE et al., 2002).

O *NormFinder* determina a estabilidade da expressão gênica pela avaliação intra e entre grupos de variação utilizando um modelo de abordagem de baixa variação, indicando uma expressão estável e, portanto, a adequação desse gene como referência (ANDERSEN et al., 2004). O *BestKeeper* mostra o valor de estabilidade dos candidatos a gene de referência individualmente, de acordo com o coeficiente de correlação de Pearson (R), sendo que valores de p mais próximos a 1,0 indicam maior estabilidade. Essa análise gera o desvio padrão médio da média do *Ct*, para que um candidato a gene de referência seja considerado estável. Além disso, o *BestKeeper Index* indica o grau de sua estabilidade pelo coeficiente R (KIRSCHNECK et al, 2016).

O método Δ*Ct* Comparativo baseia-se na comparação da expressão dos candidatos a genes de referência e cada amostra de tecido. Os genes são classificados de acordo com o desvio padrão da média dos diferentes *Ct*s do gene em questão em relação aos outros avaliados. Quanto maior a média do desvio padrão, menor a estabilidade gênica. Se os valores de *Ct* entre os genes variam em diferentes amostras de tecido, o nível de expressão gênica de cada um também varia. Quanto menor a variabilidade, maior a estabilidade dos genes (SILVER et al., 2006).

Nenhum dos algoritmos é o ideal, pois cada um utiliza um modelo matemático diferente. Por isso, recentemente, o *RefFinder* (http://www.leonxie.com/referencegene.php) foi

desenvolvido com o objetivo de fornecer um meio conveniente e adequado para avaliar os candidatos a gene de referência. O *RefFinder* utiliza os resultados dos quatro algoritmos citados anteriormente e indica o melhor gene de referência (XIE et al., 2012). Vandesompele e colaboradores (2002) relatam a necessidade de realizar a normalização com mais de um gene de referência, o que traz maior confiabilidade e acurácia à pesquisa.

2.3 Genes de referência eleitos para o processo de validação

Os genes que codificam proteínas responsáveis por funções básicas na vida do animal são normalmente selecionados para normalizarem a reação de RT-qPCR. Em ratos, o gene de referência mais utilizado é o *GAPDH* (BAJ et al., 2013; KHAN et al., 2016; SUENAGA et al., 2013). Os genes com funções celulares essenciais não possuem variação na sua expressão nos diferentes tecidos animais, em condição de higidez. Entretanto, a depender dessas condições e da espécie, estudos recentes mostram que ocorrem variações e outros genes foram estudados, sendo indicados como mais estáveis (CHANG et al., 2010; BRATTELID et al., 2010; BUSTIN et al., 2013)

O gene *GAPDH* (gliceraldeído-3-fosfato desidrogenase) está envolvido com a sexta fase da glicólise e o gene *ACTB* (β-actina) codifica uma proteína envolvida na motilidade, integridade e estruturação celular (DE MOURA et al., 2014). Em tecido neuronal de ratos saudáveis, ambos os genes citados foram os que apresentaram maior flutuação em sua estabilidade segundo o estudo de Mathur e colaboradores (2015), analisados pelo algoritmo *NormFinder*. Nesse mesmo estudo, tanto o algoritmo *geNorm* quanto o *NormFinder* acusaram os genes *TFRC* (receptor transferrina) e *B2M* (microglobulina β-2) como os mais estáveis, enquanto o *GAPDH* permaneceu como gene mais instável. Isso demonstra a importância da pré-validação dos genes de referência antes da execução da pesquisa, sendo o *GAPDH* e a *ACTB* genes não recomendados para a avaliação de neurônios em ratos, por exemplo (MATHUR et al., 2015). Outra pesquisa, em tecido de mucosa oral de ratos, também revelou que os genes *GAPDH* e *ACTB* estavam significativamente alterados em animais com carcinomas de células escamosas quando comparados aos animais saudáveis, reiterando a informação de que, a depender da situação experimental, os genes tidos como "de referência" podem ter sua expressão alterada (PENG & MCCORMICK, 2016).

Na glicólise, a glicose é oxidada e produz duas moléculas de piruvato e duas de ATP. A sexta etapa da glicólise forma o NADH e uma molécula de água: o GAPDH é oxidado, formando um ATP. Ocorre a liberação da coenzima reduzida NADH e, por estar em quantidade

limitada nas células, ele será reoxidado a NAD^+. Por participar de uma via metabólica essencial para as células de um mamífero, o *GAPDH* é um gene de expressão contínua no organismo dos seres vivos saudáveis, sendo considerado um candidato a gene de referência para as reações em cadeia da polimerase quantitativa em tempo real (STANFIELD, 2015). Além disso, é reconhecido como uma proteína multifuncional que age na transcrição, em fatores pós transcricionais, no transporte transmembrana e na apoptose de células sob estresse oxidativo (NAKAJIMA et al., 2017).

Em relação aos genes *RPL32* (proteína ribossomal L32) e *RPL4* (proteína ribossomal L4), o ribossomo é uma organela que catalisa a síntese proteica e é constituído de duas subunidades, sendo a pequena 40S e a grande 60S. Essas subunidades são compostas de 4 tipos de RNA, que codificam 80 proteínas estruturais diferentes. O *RPL32* e o *RPL4* são genes que codificam proteínas da subunidade 60S, sendo também essenciais para a manutenção da vida (SIENNA et al., 2000). Dessa forma, são candidatos a gene de referência, sob validação da estabilidade de sua expressão.

O *POLR2A* codifica a proteína RNA polimerase 2 da subunidade A. É um gene expresso em 11 tecidos dos mamíferos, sendo eles: glândula adrenal, cérebro, coração, rim, fígado, pulmão, músculos, baço, timo, testículos e útero. Esse gene participa da transcrição de todos os genes codificadores de proteínas nas células eucariotas, processo fundamental para a vida. A perda do gene *POLR2A* é incompatível com a vida (CLARK et al., 2016). Dessa forma, o *POLR2A* é um candidato a gene de referência mediante verificação de sua estabilidade nas amostras em questão.

Os genes *RPL32*, *RPL4*, *GAPDH* e *POLR2A* foram os mais estáveis na detecção de insuficiência cardíaca pós infarto em ratos segundo Brattelid e colaboradores (2010). O *GAPDH* manteve expressão constante em cardiomiócitos de ratos, porém essa foi diminuída nas células estimuladas eletricamente (BRATTELID et al., 2010), o que reitera a necessidade de realizar a validação desse candidato a gene de referência antes do início das análises.

Não foram encontrados estudos sobre a validação de genes de referência em tecido cardíaco de ratos induzidos à obesidade e ao diabetes. Caso a normalização gênica seja realizada com a utilização de genes instáveis haverá um impacto na quantificação da expressão gênica, podendo prejudicar o resultado final. Dessa forma, a necessidade de validar a estabilidade dos genes de referência tornou-se evidente. Encontrar o gene de referência que melhor normaliza uma reação é crucial na aplicação da técnica de RT-qPCR (NISHIMURA et al., 2006).

2.4 A obesidade e o diabetes

A obesidade é uma doença multifatorial caracterizada pelo aumento do peso corporal associado à inflamação crônica, devido a um aumento da produção de citocinas pelos adipócitos, que comprometem a sensibilidade do organismo à insulina (CEFALU et al., 2015). Normalmente relacionado à ingestão calórica desequilibrada e uma dieta desbalanceada, o diabetes *mellitus* tipo 2 frequentemente está associado à obesidade e ao sedentarismo, apresentando basicamente, três problemas: resistência tecidual à ação da insulina; problemas na secreção desse hormônio, gerando aumento da glicemia; e aumento da produção de glicose pelo fígado. Além da ingestão calórica excessiva, alguns fatores sobrecarregam as células β pancreáticas, como puberdade, gravidez, sedentarismo, podendo os indivíduos com diabetes *mellitus* tipo 2 ser assintomáticos por muitos anos (ADA, 2017). A obesidade e o diabetes *mellitus* tipo 1 são fatores que predispõe a ocorrência da síndrome metabólica, caracterizada por uma junção de fatores que acarretam problemas cardíacos e diabetes *mellitus* tipo 2 (WATANABE et al., 2017).

Tanto a hiperlipidemia quanto a hiperglicemia podem culminar no estresse oxidativo. A hiperlipidemia causa uma modificação na membrana das células gerando um desequilíbrio na osmolaridade celular e no colesterol em LDL. Ademais, o aumento do volume dos adipócitos afasta os receptores de insulina nessas células, colaborando para a ocorrência da hiperglicemia. Essa, no momento de transformar o excesso de glicose que entra na célula em sorbitol e, posteriormente, em frutose, gera um aumento de NADH e diminuição de NAD^+, culminando no acúmulo das espécies reativas de oxigênio (STANFIELD, 2015). Com o acúmulo de EROs ocorre o aumento da ativação de mastócitos, células de defesa intimamente relacionadas à ocorrência de fibrose no miocárdio. Os mastócitos possuem grânulos que contém proteases, como triptase e quimase (OKAMURA et al., 2017). Com o estímulo à apoptose causado pelas EROs, a estrutura cardíaca é comprometida, pois as lacunas geradas pela morte programada de cardiomiócitos é preenchida por tecido fibroso. Dessa forma, a obesidade e o diabetes mellitus podem acarretar disfunções que resultarão na insuficiência cardíaca (SHUCKLA et al., 2017).

REFERÊNCIAS

ADA - AMERICAN DIABETES ASSOCIATION. **Classification and diagnosis of diabetes**. Diabetes Care. v. 40, n. 1, p. 11-14, 2017.

AGRIMONTI, C.; SANANGELANTONI, A. M.; MARMIROLI, N.; **Simultaneous enumeration of *Campylobacter jejuni* and *Salmonella enterica* genome equivalents by melting curve analysis following duplex real time PCR in the presenceof SYBR Green.** Food Science and Technology. v. 1, n. 1, p. 1-25, 2018.

ANDERSEN, C. L.; JENSEN, J. L.; ORNTOFT, T. F. **Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation aproach to identify genes suited for normalization, aplied to bladder and colon cancer data sets.** Cancer Research. v. 64, n. 1, p. 5245–5250, 2004.

BAJ, G. et al. **Regulation of the spatial code for BDNF mRNA isoforms in the rat hipocampus following pilocapine-treatment: a systematic analysis using laser microdissection and quantitative real-time PCR.** Hipocampus. v. 23, n. 1, p. 413-423, 2013.

BRATTELID, T. et al. **Reference gene alternatives to *Gapdh* in rodent and human heart failure gene expression. studies.** Molecular Biology. v. 11, n. 22, p. 1-10, 2010.

BUSTIN, S. A. et al. **The need for transparency and good practices in the qPCR literature.** Nature. v. 10, n. 1, p. 1063-1067, 2013.

CEFALU, W. T. et al. **Advances in science, treatment, and prevention of the disease of obesity: reflections from a *Diabetes Care* editors' expert forum.** Diabetes Care. v. 38, n. 8, p. 15676-1582, 2015.

CHANG, M. M.; LI, A.; FEISSNER, R.; AHMAD, T. **RT-qPCR demonstrates light-dependent AtRBCS1A and AtRBCS3B mRNA expressions in *Arabidopsis thaliana* leaves.** Laboratory Exercises. v. 44, n. 4, p. 405-411, 2016.

CHEN, X. **Identification of reference genes and miRNAs for RT-qPCR in testosterone propionate-induced Bening prostatic hyperplasia in rats.** Andrologia. v. 1, n. 1, p. 1-6, 2017.

CLARK, V. E. et al. **Recurrent somatic mutations in POLR2A define a distinct subset of meningiomas.** Nature Genetics. v. 48, p. 1253-1259, 2016.

DE MOURA, A. C.; LAZZARI, V. M.; DA VEIGA, A. B. G. **Transcriptional expression study in the central nervous system of rats: what gene should be used as internal control?** Einstein. v. 12, n. 3, p. 336-341, 2014.

DO LIVRAMENTO K. G. et al. **Gene expression profile analysis is directly affected by the selected reference gene: the case of leaf-cutting *Atta sexdens*.** Insects. v. 1, n. 1, p. 1-16, 2018.

DUBOIS, B. et al. **Maternal low-protein diet alters the expression of real-time quantitative polymerase chain reaction reference genes in and age-, sex-, and organ-dependent manner in rat offspring.** SciVerse Science Direct. v. 33, n. 1, p. 235-241, 2013.

FREITAS N. C. et al. **Validation of reference genes for qPCR analysis of *Coffea arabica* L. somatic embryogenesis-related tissues.** Plant Cell, Tissue and. Organ Culture. V. 128, n. 1, p. 663-678, 2017.

GANOPOULOS, I. et al. **A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese.** Food Chemistry. v. 141, n. 1, p. 835-840, 2013.

IYER, G. et al. **Identification of stable housekeeping genes in response to ionizing radiation in cancer research.** Nature Scientific Reports. v. 7, n. 1, p. 1-9, 2017.

KHAN, H. A. et al. **Comparative evaluation of immunohistochemistry and real-time PCR for measuring proinflammatory cytokines gene expression in livers of rats treated with gold nanoparticles.** Experimental and Toxicology Pathology. v. 1, n. 1, p. 1-10, 2016.

HE, Y. et al. **Methamphetamine alters reference gene expression in nigra and striatum of adult rat brain.** Neurotoxicology. v. 29, n. 1, p. 138-145.
KOSINOVÁ, L. et al. **Reference genes in rat pancreatic islets early after isolation affects results of gene expression. studies.** Plos One. v. 11, n. 4, p. 1-18, 2016.
LEE, K. S.; et al **Validation of commonly used reference genes for sleep-related gene expression studies.** BMC Molecular Biology. v. 10, n. 1, p. 10-45, 2009.
LI, X. et al. **NMR-based metabonomic and quantitative real-time PCR in the profiling of metabolic changes in carbono tetrachloride-induced rat liver injury.** Journal of Pharmaceutical and Biomedical Analysis. v. 89, n. 1, p. 42-49, 2014.
MATHUR, D. et al. **Bypassing hazard of housekeeping genes: their evaluation in rat granule neurons treated with cerebrospinal fluid of multiple sclerosis subjects.** Frontiers in Cellular Neuroscience. v. 1, n. 1, p. 1-31, 2015.
NAKAJIMA, H. et al. **Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) aggregation causes mitochondrial disfunction during oxidative stress-induced cell death.** Jounal of Biological Chemistry. v. 292, n. 11, p. 4727-4742, 2017.
NISHIMURA, M. et al. **Effects of prototypical drug-metabolizing enzyme inducers on mRNA expression. of housekeeping genes in primary cultures of human and rat hepatocytes.** Biochemical and Biophysical Research Communications. v. 346, n. 1, p. 1033-1039, 2006.
NOLAN, T.; HANDS, R. E.; BUSTIN, S. A. **Quantification of mRNA using real-time RT-PCR.** Nature Protocols. v. 1, n. 1, p. 1559–1582, 2006.
OKAMURA, K. et al. **Positive correlation between blood pressure or heart rate and chymase-dependent angiotensin Il-forming activity in circulating mononuclear leukocytes measure by new ELISA.** Clinical and Experimental Hypertension. v. 1, n. 1, p. 1-7, 2017.
PENG, X.; MCCORMICK, D. L. **Identification of reliable reference genes for quantitative gene expression. studies in oral squamous cell carcinomas compared to adjacent normal t-issues in the F344 rat model.** Oncology Reports. v. 36, n. 1, p. 1076-1084, 2016.
PFAFFL M. W. **The ongoing evolution of qPCR.** Methods. v. 50, n. 1, p. 215–216, 2010.
SAMBROOK, J. F. e RUSSEL, D. W. **Molecular Cloning**. vol. 1, 3. ed. Nova York – USA, Cold Spring Harbor Laboratory Press, 2002.
SHUCKLA, S. K. et al. **HMGCS2 is a key to ketogenic enzyme potentially involved in type 1 diabetes with high cardiovascular risk.** Nature Scientific Reports. v. 7, n. 4590, p. 1-10, 2017.
SIENNA, N.; LARSON, D. E.; SELLS, B. H. **Dexamethasone stimulates ribosomal protein L32 gene transcription at rat myoblast.** Mollecular and Cellular Endocrinology. v. 167, n. 1-2, p. 127-137. 2000.
SILVER, N.; et al. **Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR.** BMC Molecular Biology. v. 7, n. 33, p. 1-9, 2006.
SMITH, C. J.; OSBORN, A. M. **Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology**. Federation of European Microbiological Societies. v. 67, n. 1, p. 6-20, 2008.
STANFIELD, C. L. **Fisiologia humana**. 5. ed. São Paulo, SP: Pearson, 2015.
SUENAGA, K. et al. **Differential gene expression profiling between genotoxic and non-genotoxic hipatocarcinogens in young rat liver determined by quantitative real-time PCR and principal componente analysis.** Mutation Research. v. 751, n. 1, p. 73-83, 2013.
VANDESOMPELE, J. et al. **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** Genome Biology. v. 3, n. 1, p. 1-9, 2002.

WANG, Y. et al. **Identification of endogenous reference genes for RT-qPCR analysis of plasma microRNAs levels in rats with acetaminophen-induced hepatotoxicity.** Aplied Toxicology. v. 33, n. 1, p. 1330-1336, 2013.

WATANABE, S. et al. **Daily coffee intake inhibits pancreatic *β*-cells damage and nonalcoholic steatohepatitis in mouse model of spontaneous metabolic syndrome, Tsumura-Suzuki obese diabetic mice.** Metabolic Syndrome and Related Disorders. v. 15, n. 4, p. 1-8, 2017.

WEYRICH, A.; AXTNER, J.; SOMMER, S. **Selection and validation of reference genes for real-time RT-PCR studies in the non-model species *Delomys sublineatus*, na endemic Brazilian rodent.** Biochemical and Biophysical Research Communications. v. 392, n. 1, p. 145-149, 2010.

XIE, F. et al. **miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs.** Plant Molecular Biology. v. 80, n. 1, p. 75-84, 2012.

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ARTICLE 1 Validation of reference genes for RT-qPCR in cardiac tissue of obesity and diabetes induced rats

VALIDATION OF REFERENCE GENES FOR RT-qPCR IN CARDIAC TISSUE OF OBESITY AND DIABETES INDUCED RATS

Marina Martins de Oliveira[1*]; Kalynka Gabriella do Livramento[2]; Maísa Lamounier Magalhães[3]; Luciano Vilela de Paiva[2]; Ana Paula Peconick[1]

[1]Federal University of Lavras, Veterinarian Medicine Department [*]mimartins140140@gmail.com
[2] Federal University of Lavras, Chemistry Department
[3] Federal University of Lavras, Food Science Departament

ABSTRACT

The present study aims 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 hearts of the animals were collected and the cardiac apex was assigned to the molecular techniques for RNA extraction, performed with TRIzol®. Candidates for reference genes were *GAPDH*, *POLR2A*, *RPL32*, and *RPL4*. The RT-qPCR technique was performed in propper 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 target gene used to verify the differences in gene expression of candidates for reference genes was *CMA1*, responsible for the traduction of chymase enzyme. 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. 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. The *GAPDH* and *POLR2A* genes are the best to normalize the reactions with the samples submitted to obesity by the hyperlipidic diet and diabetes.

Key words: Normalization, RT-qPCR, RefFinder, *GAPDH*, *POLR2A*

INTRODUCTION

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

The specie *Rattus norvegicus albinus* is one of the most widely used animal models in experiments with metabolic alterations (Hunt et al., 2017). Some of these changes can cause serious heart problems and it is of interest to assess the timing of these changes. Altering the gene expression of certain genes may precede the histological or clinical changes in these animals (Khalilpourfarshbafi et al., 2017). For this, 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, using the real-time quantitative polymerase chain reaction (RT- qPCR) (do 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). The increase in 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, provided that both the reference gene and target genes can be quantified in the same sample (Zhu et al., 2014).

Reference genes are selected by choosing genes that maintain their constant expression in a given situation. RefFinder (East Carolina University, Greenville, NC, USA) 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 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

2.1 – Biological samples

The samples were obtained from rats 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. Initially, 18 animals were divided into 3 groups, which were: obese animals (D), obese and diabetic animals (E) and control animals (G). All groups were kept isolated in metabolic cages arranged vertically in 4 cages and horizontally in 6 cages. The room was heated, with a temperature 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 experimente: streptozotocin was used in a single dose of 45mg/kg *per* intraperitoneal route. Diabetes was proven by measuring blood glucose 72 hours after induction, and blood was collected by cutting the tip of the tail. Blood glucose greater than 300mg/dL confirms 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 experimente, to reach the 42nd day, obese. All animals were recieving 40g of feed per day. Obesity was proven through the Lee Index analysis (Panveloski-Costa et al., 2011). Euthanasia was performed on the 70th day of the experiment. The animals were induced with isoflurane by inhalation and euthanized in guillotine. The chest was opened, the heart was collected, and the cardiac apices were collected and frozen in liquid nitrogen to be assigned to the molecular biology technique.

2.2 – RNA extraction and cDNA confection

The cardiac apexes 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.

Total RNA was extracted using 0.5ml TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and 200µL of formalin, followed by centrifugation at 10,000g for 10 minutes. The supernatant was collected and 100µL of isopropyl alcohol was added, this solution being kept in a freezer for a minimum of 40 minutes and a maximum of 2 hours. The samples were again centrifuged at 12,000g for 20 minutes when RNA pellet formation occurred. This pellet was washed with 75% ethyl alcohol and dried in a concentrator. The pellet was resuspended in RNase-free water and stored at -80°C. 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. The RNA concentration of each sample was measured by the Nanodrop 1000 Spectophotometer (ThermoFisher Scientific, Waltham, MA, USA). The purity of the RNA was 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. 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. The cDNA 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

Four candidate gene reference genes were selected according to the best results previously published (Brattelid et al., 2010), glyceraldehyde-6-phosphate dehydrogenase (*GAPDH*), ribosomal protein L32 (*RPL32*), ribosomal protein L4 (*RPL4*), RNA polymerase II subunit A (*POLR2A*).

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/). In BLAST it was possible to find the start codon and stop codon. The sequence was copied into OrfFinder, which results in a protein sequence. This sequence was sent to the conserved domain analysis, a region in which the primer should not be drawn. The sequence outside the conserved domain region was copied to the OligoPerfect software, in which the number of base pairs should be 20, the difference in annealing temperature

between the forward primer and the reverse primer should be less than 3°C and the percentage of bases C and G should be between 45% and 60%. The chosen primer sequences were copied to the OligoAnalyzer, software that allows analyzing the amount of hairpins, self-dimers and hetero-dimers of the primers in question, considering that the lower the number of hairpins, the better, while the self-dimmers and hetero-dimers must be greater than -6.

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	**Número de acesso**	**Sequência dos *primers* (Fw/Rv 5'-3')**	***Tm* (°C)**	***Amplicon* (pb)**	**E (%)**	**R^2**
GAPDH	NP_058704.1	GCCCAGCAAGGATACTGAGA GGATGGAATTGTGAGGGAGA	68.53 66.51	103	85	0,997
RPL32	NM_013226.2	GCCCAAGATCGTCAAAAAGA AATCTTCTCCGCACCCTGTT	65.51 69.53	120	92	0,998
RPL4	BC081801.1	GCCAAATCGGAGAAGATTGT TGCAGGCTTCTTCAGCTTCT	65.50 69.54	108	92	0,979
POLR2A	XM_001079162.5	TCTCCCACTTCTCCTGGCTA CTCCTCATCGCTGTCTTCTG	69.53 67.52	87	95	0,991
CMA1	NC_005100.4	GTGTCTGCTGCTCCTTCTCC CGGGAGTGTGGTATGCACT	70.55 68.54	81	81	0,977

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) 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. The annealing and extension were 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 (Ct) at the cardiac apex of rats under the following conditions: obese; obese and diabetic; control or healthy animals; and a pool of all samples. The values of Ct 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. The RefFinder tool was used to access the stability of gene expression in cardiac tissue samples.

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. Vn/n + 1 is calculated between each set of two consecutive normalization factors (FN), starting with the relative expression of values from two more stable genes, as shown by RefFinder. The calculation consists of the ratios of the log-transformed NFs of each sequential combination of two NFs. The standard deviation of the analysis data for each combination of FN is calculated. 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.

3.0 – 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. The efficiency (E) of the RT-qPCR reaction and the determination coefficient (R^2) were calculated using the LinReg software for each primer pair. 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 Table 2.

Table 2. Mean expression. of the reference genes candidates and the variation between the most and the least expressed among the diferentes groups of samples of the experiment.

Genes	D	E	G	Pool	Variation
	Ct	***Ct***	***Ct***	***Ct***	***ΔCt***
GAPDH	20,86	21,74	20,32	18,87	7,660
POLR2A	28,62	29,08	27,43	27,12	5,6
RPL32	25,68	24,6	23,68	24,66	14,2
RPL4	23,980	24,56	23,76	21,78	10,59

Ct: *Cycle Threshold*. *ΔCt:* difference between the sample that most expressed the gene in question and the least expressed. "D", "E" and "G" correspond to the treatment groups, being obese, obese and diabetic and control, respectively.

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, ΔCt Comparative 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 3. Boxplot diagrams with variation levels of reference gene candidates are shown in Figure 1.

Table 3. *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
D x G										
GAPDH	0,986	1	1,033	1	1,535	1	1,000	1	1,000	1
POLR2A	0,986	2	1,597	3	1,634	2	1,861	2	1,861	2
RPL32	2,144	4	2,068	4	3,312	4	3,722	4	3,722	4
RPL4	1,840	3	1,421	2	3,264	3	2,913	3	2,913	3
E x G										
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
D x E										
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

Samples of the cardiac apex of each group of animals with metabolic disturbance, indicated in the table. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L32 (*RPL32*), ribosomal protein L4 (*RPL4*), and RNA polymerase II subunit A (*POLR2A*). 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 Ct of the samples; OSV: overall stability value, value of the final ranking, given by RefFinder.

Boxplot diagrams with variation levels of reference gene candidates are shown in Figure 1.

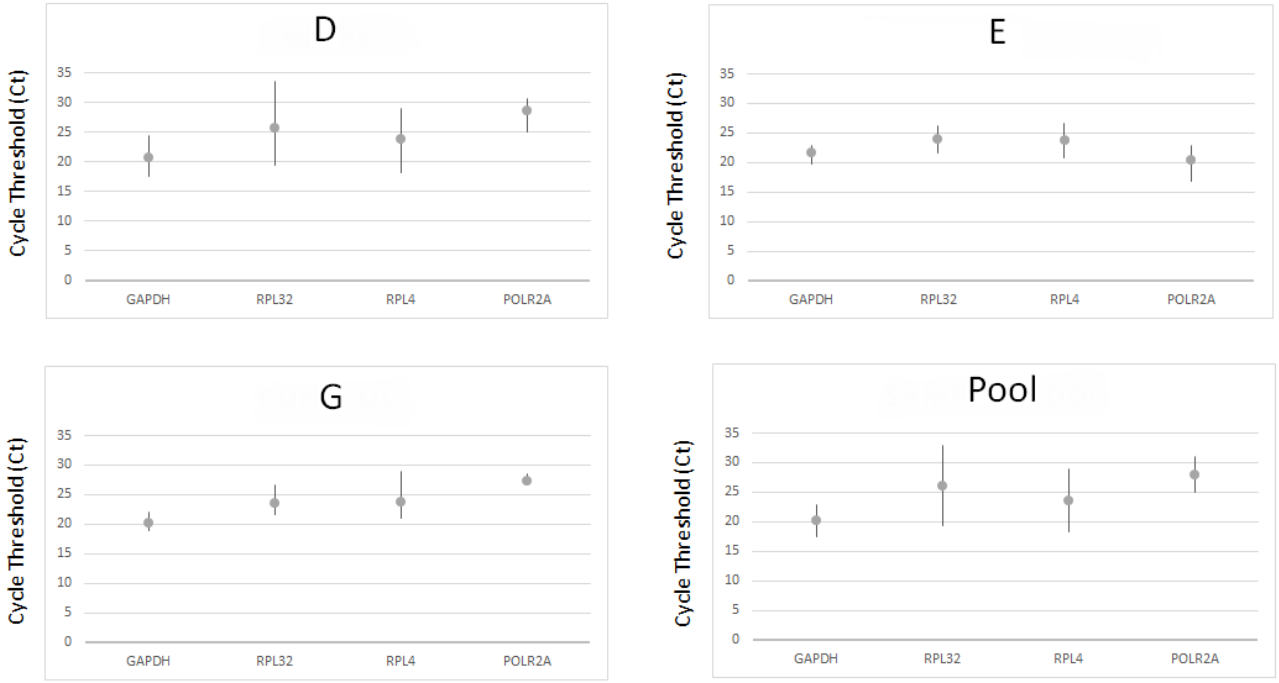

Figure 1. Expression of reference gene candidates determined by quantification of cycles (Ct) in sample groups. The bars indicate the maximum and minimum values of the Cts while the circles represent the mean values. “D” means obese group; “E” means obese and diabetic group; “G” means control group;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

3.3- Determination of the optimum number of reference genes

For the gene expression analysis of obese animals compared to healthy, 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 4.

Table 4. 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**			
	D+G	**D+E**	**E+G**	**D+E+G**
V2/3	0,4723	0,4927	0,1472	0,4041
V3/4	0,6309	0,4085	0,5285	0,5248

Being "D + G" obese compared to the control group; "D + E" obese compared to obese and diabetic; "E + G" obese and diabetic compared to control; and "D + E + G", the sample pool.

3.4 – Validation of the selected reference genes

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. Expression was dependent on the reference genes used for normalization. *CMA1* levels were overestimated when reference genes not suitable for normalization were used. The *CMA1* 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 1. 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 4.

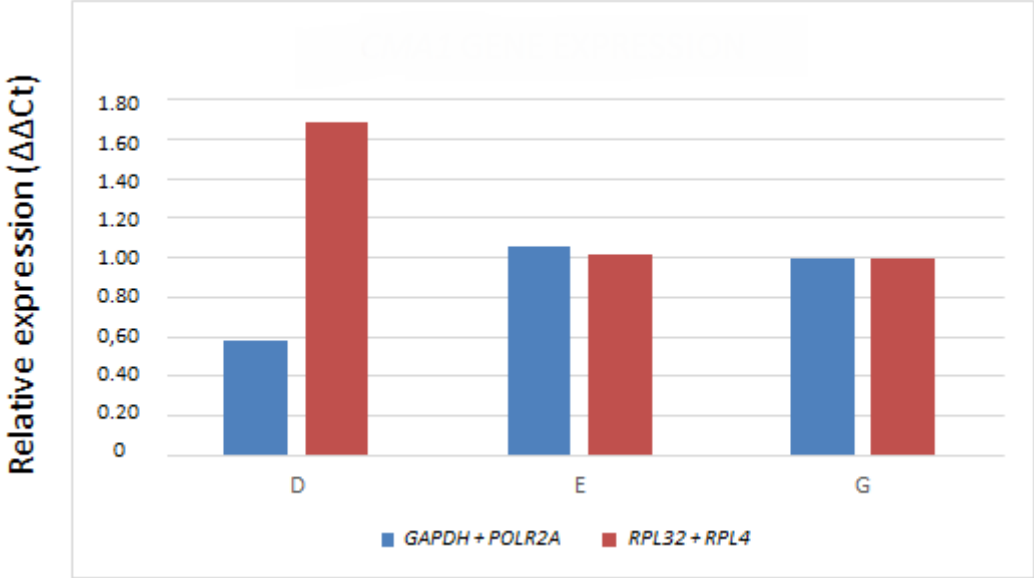

Figure 3. Differences in the gene expression of *CMA1* using the selected reference genes. Quantification of relative gene expression was done with two strategies: the combination of the two most stable reference genes, *GAPDH* + *POLR2A*, and the two less stable genes, *RPL4* and *RPL32*. The columns represent the gene expression of the two combinations in each treatment, being: "D", obese; "E", obese and diabetic; and "G", control. The relative expression refers to the standard deviation of the differences between the largest and the smallest Ct of the samples.

4.0 – 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, 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 Ct 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, *GAPDH* was the second most unstable gene among all studied. 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. 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 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 apex. However, each experimental situation and each tissue in question must be previously validated.

In relation to obese and diabetic rats, compared to healthy animals, *GAPDH* was the gene with lower Ct 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, *GTPDH* Ct 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 gene *GAPDH* 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). In this way, 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., 2006, 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. The validation of the genes was based on the expression profile of the *CMA1* gene. 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.

5.0 – Conclusion

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.

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REFERÊNCIAS

Ahmad S, Voncannon JL, Bader M, Varagic J, Ferrario CM. Chymase – mediated angiotensin II formation in the heart of rats overexpressing the human angiotensin gene. *Hypertens*. 64: 1-12, 2014.

Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation aproach to identify genes suited for normalization, aplied to bladder and colon cancer data sets. *Can. Res.* 64: 5245–5250, 2004.
doi: 10.1158/0008-5472

Brattelid T, Winer LH, Levy FO, Liestol K, Sejersted OM, Andersson KB. Reference gene alternatives to *GAPDH* in rodend and human heart failure gene expression. studies. *BMC Molec. Biol*. 11: 1-10, 2010.
doi: 10.1186/1471-2199-11-22

Bortolin RH, Neto FPF, Arcaro CA, Bezerra JF, Da Silva FS, Ururahy MAG, Souza KSC, Lima VMGDM, Luchessi AD, Lima FP, Fook MVL, Da Silva BJ, Almeida MG, Abreu BJ, Rezende LA, Rezende AA. Anabolic effect of insulin therapy on the bone: *osteoprotegerin* and *osteocalcin* up-regulation in streptozootocin-induced diabetic rats. *Basic & Clinical Pharmacology & Toxicology*. 120: 227-234, 2017.
doi: 10.1111/bcpt.12672

Bustin SA, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley G, Wittwer CT, Schjerling P, Day PJ, Abreu M, Aguado B, Beaulieu JF, Beckers A, Bogaert S, Browne JA, Carrasco-Ramiro F, Ceelen L, Cibrowski K, Cornillie P, Coulon S, Cuypers A, Brouwer SD, Ceuninck LD, Craene JD, DeNaeyer H, Spiegelaere WD, Deckers K, Dheedene A, Durinck K, Ferreira-Teixeira M, Fieuw A, Gallup JM, Gonzalo-Flores S, Goossens K, Heindryckx F, Herring E, Hoenicka H, Icardi L, Jaggi R, Javad F, Karampelias M, Kibenge F, Kibenge M, Kumps C, Lambertz I, Lammens T, Markey A, Messiaen P, Mets E, Morais S, Mudarra-Rubio A, Nakiwala J, Nelis H, Olsvik PA, Pérez-Novo C, Plusquin M, Remans T, Rihani A, Rodrigues-Santos P, Rondou P, Sanders R, Schimidt-Bleek K, Skovgaard K, Smeets K, Tabera L, Toegel S, Acker TV, Broeck WV, Meulen JV, Gele MV, Peer GV, Poucke MV, Roy NV, Vergult S, Wauman J, Tshuikina-Wiklander M, Willems E, Zaccara S, Zeka F, Vandesompele J The need for transparency and good practices in the qPCR literature. *Nature*. 10: 1063-1067, 2013.

Cefalu WT, Bray GA, Home PD, Garvey WT, Klein S, Pi-Sunyer FX, Hu FB, Raz I, Van Gaal L, Wolfe BM, Ryan DH. Advances in science, treatment, and prevention of the disease of obesity: reflections from a *Diabetes Care* editors' expert forum. *Diab. Care*. 38: 1567-1582, 2015.

Chang Z, Ling C, Yamashita M, Welham NV. Microarray-driven validation of reference genes for quantitative real-time polymerase chain reaction in a rat vocal fold model of mucosal injury. *Analyt. Biochem*. 406: C214-C221, 2010.

Chen X. Identification of reference genes and miRNAs for RT-qPCR in testosterone propionate-induced Bening prostatic hyperplasia in rats. *Androl*. 1: 1-6, 2017.
doi: 10.1111/and.12966

Daneshpajooh M, Bacos K, Bysani M, Bagge A, Laakso EO, Vikman P, Eliasson L, Mulder H, Long C. *HDAC7* is overexpressed in human diabetic islets and impairs insulin secretion in rat islets and clonal beta cells. *Diabetol*. 60: 116-125, 2017.
doi: 10.1007/s00125-016-4113-2

Do Livramento KG, Freitas NC, Máximo WPF, Zanetti R, Paiva LV. Gene expression profile analysis is directly affected by the selected reference gene: the case of leaf-cutting *Atta sexdens*. *Insec*. 1: 1-16, 2018.
doi: 10.3390/insects9010018

Eissa N, Kermarred HH, Bernstein CN, Ghia JE. Apropriateness of reference genes for normalizing messenger RNA in mouse 2,4-dinitrobenzene sulfonic acid (DNBS) - induced colitis using quantitative real time PCR. *Sci. Rep*. 7: 1-13, 2017.

Farnsworth CW, Schott EW, Benvie A, Kates SL, Schwarz EM, Gill SR, Zuscik MJ, Mooney RA. Exacerbated *Staphylococcus aureus* foot infection in obese/diabetic mice are associated with impaired germinal center reactions, Ig class switching, and humoral immunity. *J. Immunol*. 1: 1-14, 2018.
doi: 10.4049/jimmunol.1800253

Fedoseeva LA, Shevelev OB, Kolosova NG, Dymshits GM. MS2 phage ribonucleoproteins as exogenous internal control for RT-qPCR data normalization in gene expression. study of developing rat brain. *Biochem*. 79: 706-716, 2014.

doi: 10.1134/S0006297914070128

Ferreira ECS, Bortolin RH, Freire-Neto FP, Souza KSC, Bezerra JF, Ururahy MAG, Ramos AMO, Himelfarb ST, Abreu BJ, Didone TVN, Pedrosa LFC, Medeiros AC, Doi SQ, Brandão-Neto J, Hirata RDC, Rezende LA, Almeida MG, Hirata MH, Rezende AA. Zinc suplementation reduces *RANKL*/*OPG* ratio and prevents boné architecture alternations in ovariectomized and type 1 diabetic rats. *Nutri. Res*. 40: 48-56, 2017.
doi: 10.1016/j.nutres.2017.03.004

Hunt HB, Pearl JC, Diaz DR, King KB, Donnelly E. Bone tissue collagen maturity and mineral content increase with sustained hyperglicemia in the KK-Ay murine modelo f type 2 diabetes. *J. Bon. Min. Res*. 33: 921-919, 2017.
doi: 10.1002/jbmr.3365

Julian GS, Oliveira RW, Tufik S, Chagas JR. Analysis of the stability of housekeeping gene expression in the left cardiac ventricle of rats submitted to chronic intermittent hypoxia. *J. Bras. Pneum*. 42: 1-4, 2016.
doi: 10.1590/S1806-37562015000000133

Khalilpourfarshbafi M, Hajiaghaalipour F, Selvarajan KK, Adam A. Mesenchymal stem cell-based therapies against podocyte damage in diabetic nephropathy. *J. Tis. Eng. Regen. Med*. 14: 201-210, 2017.
doi: 10.1007/s13770-017-0026-5

Kirschneck C, Proff P, Fanghänel J, Wolf M, Roldán JC, Römer P. Reference genes for valid gene expression studies on rat dental, periodontal and alveolar bone tissue by means of RT-qPCR with a focus on orthodontic tooth movement and periodontitis. *An. Anatom*. 204: 93-115, 2016.
doi: 10.1016/j.aanat.2015.11.005

Lambert K, Hokayem M, Thomas C, Fabre O, Cassan C, Bourret A, Bernex F, Feuillet-Coudray C, Notarnicola C, Mercier J, Avignon A, Bisbal C. Combination of nutritional polyphenols suplementation with exercise training counteracts insulin resistance and improves endurance in high-fat diet-induced obese rats. *Nat. Sci. Rep*. 8: 1-10, 2018.

Lerco MM, Spadella CD, Machado JLM, Schellini AS, Padovani CR. Caracterização de um modelo experimental de diabetes mellitus, induzido pela aloxana em ratos. Estudo clínico e laboratorial. *Acta Cir. Bras.* 18: 132-142, 2003

Li X, Zhang F, Wang D, Li Z, Qin X, Du G. NMR-based metabonomic and quantitative real-time PCR in the profiling of metabolic changes in carbono tetrachloride-induced rat liver injury. *J. Pharm. Biomed. Anal*. 89: 42-49, 2014.
doi: 10.1016/j.jpba.2013.10.023

Mathur D, Urena-Peralta JR, Lopez-Rodas G, Casanova B, Coret-Ferrer F, Burgal-Marti M. Bypassing hazard of housekeeping genes: their evaluation in rat granule neurons treated with cerebrospinal fluid of multiple sclerosis subjects. *Front. Cell. Neurosci*. 1: 1-31, 2015.
doi: 10.3389/fncel.2015.00375

Marcioli MAR, Silva JLC, Ribeiro LFC, Brancalhão RMC, Bertolini GRF. Neurotrophin expression and histomorphometric evaluation in Wistar rats subjected to neural mobilization after compression of the median nerve. *Rev. Bras. Ortop. (Eng Vers).* 53: 276-280, 2018.
doi: 10.1016/j.rboe.2018.03.006

Nagao K, Jinnouchi T, Kai S, Yanagita T. Pterostilbene, a dimethylated analog of resveratrol, promotes energy metabolism in obese rats. *J. Nut. Biochem*. 43: 151-155, 2017.
doi: 10.1016/j.jnutbio.2017.02.009

Panveloski-Costa AC, Pinto-Júnior DAC, Brandão BB, Moreira RJ, Machado UF, Seraphim PM. Resistive training reduces inflammation in skeletal muscle and improves the peripheral insulin sensitivity in obese rats by hyperlipidic diet. *Arq. Bras. Endocrinol. Metab.* 55:155-163, 2011.

Petersson SJ, Christensen LL, Kristensen JM, Kruse R, Andersen M, Hojlund K. Effect of testosterone on markers of mitochondrial oxidative phosphorylation and lipid metabolism in muscle of aging men with subnormal bioavailable testosterone. *Euro. J. Endoc*. 171: 77-88, 2014.
doi: 10.1530/EJE-14-0006

Perry RJ, Peng L, Cline GW, Dufor S, Petersen KF, Shulman GI. Mechanisms by which a very-low-calorie diet reverses hyperglycemia in a rat model of type 2 diabetes. *Cell Metabol*. 27: 1-8, 2018.
doi: 10.1016/j.cmet.2017.10.004

Pfaffl MW. The ongoing evolution of qPCR. *Methods*. 50: 215-216, 2010.
doi: 10.1016/j.ymeth.2010.02.005

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 29: 45, 2001.

Rahman TU, Ullah K, Ke ZH, Guo MX, Jin LY, Zhou YZ, Cheng Y, Dong XY, Pang HY, Wang TT, Sheng JZ, Huang HF. Hypertriglyceridemia in female rats during pregnancy induces obesity in male offspring via altering hypotalamic leptin signaling. *Oncotarg*. 8: 53450-53464, 2017.

Rajkumar AP, Qvist P, Lazarus R, Lescai F, Ju J, Nyegaard M, Mors O, Borglum AD, Li Q, Christensen JH. Experimental validation of methods for differential gene expression. analysis and sample pooling in RNA-seq. *BMC Genom*. 16: 1-8, 2015.
doi: 10.1186/s12864-015-1767-y

Sambrook JF and Russel DW. Molecular Cloning. vol. 1, 3. ed. Nova York – USA, Cold Spring Harbor Laboratory Press, 2002.

Shuckla SK, Liu W, Sikder K, Addya S, Sarkar A, Wei Y, Rafig K. HMGCS2 is a key to ketogenic enzyme potentially involved in type 1 diabetes with high cardiovascular risk. *Nat. Sci. Rep*. 7: 1-10, 2017.
doi: 10.1038/s41598-017-04469-z

Si X, Zhou Z, Strape P, Blanchard C. A comparison of RS4-type resistant starch to RS2-type resistant starch in supressing oxidative stress in high-fat-diet-induced obese rats. *Roy. Soc. Chem*. 8: C232-C240, 2017.
doi: 10.1039/c6fo01225f

Silva VO, Lobato RV, Andrade EF, Macedo CG, Napimoga JTC, Napimoga MH, Messora MR, Murata RM, Pereira LJ. β-glucans (*Saccharomyces cereviseae*) reduce glucose levels and attenuate alveolar bone loss in diabetic rats with periodontal disease. *Plos One*. 1: 1-13, 2015.
doi: 10.1371/journal.pone.0134742

Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molec. Biol*. 7: 1-9, 2006.

Svingen T, Letting H, Hadrup N, Hass U, Vinggaard AM. Selection of reference genes for quantitative RT-PCR (RT-qPCR) analysis of rat tissue under physiological and toxicological conditions. *PeerJ*. 3: 1-15, 2015.
doi: 10.7717/peerj.855

Tan SC Carr CA, Yeoh KK, Schofield CJ, Davies KE, Clarke K. Identification of valid housekeeping genes for quantitative RT-PCR analysis of cardiosphere-derives cells preconditioned under hypoxia or with prolyl-4-hydroxylase inhibitors. *Molec. Biol. Rep*. 39: 4857-4867, 2012.
doi: 10.1007/s11033-011-1281-5

Vandesompele J, De Preter K, Pattyn F, Pope B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Gen. Biol*. 3: 1-11, 2002

Veres-Székely A, Pap D, Sziksz E, Jávorszky E, Rokonay R, Lipai R, Tory K, Fekete A, Tulassay T, Szabó AJ, Vannay A. Selective measurement of a smooth muscle actin: why β-actin cannot be used as a housekeeping gene when tissue fibrosis occurs. *BMC Molec. Biol*. 18: 1-15, 2017

Wang H, Da Silva J, Gabriel-Costa D, Ahmad S, Sun X, Lin M, Sudo RT, Varagic J, Ferrario C, Zapata-Sudo G, Groban L. Diastolic dysfunction after estrogen loss in linked to cardiac chymase in WKY but not SHR rats. *Hypertens*. 68: 285-291, 2016.

Zhou X, Zhang J, Haimbach R, Zhu W, Mayer-Ezell R, Garcia-Calvo M, Smith E, Price O, Kan Y, Zycband E, Zhu Y, Hoek M, Cox JM, Ma L, Kelley DE, Pinto S. An integrin antagonista (MK-0429) decreases proteinuria and renal fibrosis in the ZSF1 rat diabetic nephropathy model. *Pharmacol. Res. Perspec*. 5: 1-14, 2017.
doi: 10.1002/prp2.354

Zhu X, Yuan M, Shakeel M, Zhang Y, Wang S, Wang X, Zhan S, Kang T, Li J. Selection and evaluation of reference genes for expression analysis using RT-qPCR in the beet armyworm *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). *Plos One*, 9: 1-9, 2014.
doi: 10.1371/journal.pone.0084730