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Original Research Article

Identification of optimal housekeeping genes for examination of gene expression in bovine corpus luteum

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ABSTRACT

The selection of proper housekeeping genes for studies requiring genes expression normalization is an important step in the appropriate interpretation of results. The expression of housekeeping genes is regulated by many factors including age, gender, type of tissue or disease. The aim of the study was to identify optimal housekeeping genes in the corpus luteum obtained from cyclic or pregnant cows. The mRNA expression of thirteen housekeeping genes: *C2orf29*, *SUZ12*, *TBP*, *TUBB2B*, *ZNF131*, *HPRT1*, *18s RNA*, *GAPDH*, *SF3A1*, *SDHA*, *MRPL12*, *B2M* and *ACTB* was measured by Real-time PCR. Range of cycle threshold (C_t) values of the tested genes varied between 12 and 30 cycles, and *18s RNA* had the highest coefficient of variation, whereas *C2orf29* had the smallest coefficient. GeNorm software demonstrated *C2orf29* and *TBP* as the most stable and *18s RNA* and *B2M* as the most unstable housekeeping genes. Using the proposed cut-off value (0.15), no more than two of the best GeNorm housekeeping genes are proposed to be used in studies requiring gene expression normalization. NormFinder software demonstrated *C2orf29* and *SUZ12* as the best and *18s RNA* and *B2M* as the worst housekeeping genes. The study indicates that selection of housekeeping genes may essentially affect the quality of the gene expression results.

1. Introduction

Quantitative Real-time PCR is currently the most sensitive and powerful technique for examination of gene expression [1]. Real-time PCR is similar to classical PCR, but the addition of fluorescent dyes allows the monitoring of the increased level of PCR product in each cycle. The amount of mRNA transcript of an examined gene is determined by comparing the results to a standard curve made from serial dilutions of a known amount of cDNA [2]. To normalize gene expression data, the amount of mRNA of a tested gene is divided by the amount of mRNA of a housekeeping gene. The normalization allows to eliminate possible variations in: the quality of RNA between different samples, the yield of cDNA synthesis or PCR efficiency [3].

Housekeeping genes are involved in every cell's life [4,5]. Several studies indicate that the expression of housekeeping genes varies in different tissues [6]. The transcription of pseudogenes may increase the basic level of the gene used

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for normalization [7]. Different forms of the housekeeping gene may also be generated through alternative splicing and reduce primers annealing to the template DNA [8]. Diseases that cause chromosomal changes within a coding region of a housekeeping gene may interfere with the level of its mRNA, skewing the results [9]. The method of storage and preservation of the tissue may also affect the stability of housekeeping gene RNA [10]. It was also reported that mRNA level of different housekeeping genes depends on the age [11], gender [12], hormones [13] and drugs [9]. Therefore, the selection of optimal housekeeping genes is important for the determination of gene expression in a tested tissue.

The corpus luteum (CL) is a transient endocrine organ formed from cells of the follicle after ovulation. The CL secretes progesterone (P_4), which is necessary for the blastocyst implantation and pregnancy maintenance. It also participates in the maintenance of a quiescent uterus and ensures an appropriate intrauterine environment for embryonic growth and development [14,15]. The regulation of the CL secretory function involves the activation of several signal transduction pathways and the expression of many genes [16]. The aim of the study was to identify optimal housekeeping genes in the corpus luteum obtained from cyclic or pregnant cows.

2. Materials and methods

2.1. *Corpora lutea collection*

Corpora lutea from cyclic cows and heifers were harvested within 20 min following sacrifice from commercial slaughterhouse. Two CLs from each of the four stages of the estrous cycle (days 1–5, 6–10, 11–16 and 17–20) and three stages of early pregnancy (weeks 3–5, 6–8 and 9–12) were collected for the experiment. Stages of the CL were estimated according to Ireland et al. [17] and Jainudeen and Hafez [18]. Immediately after collection, the CLs were frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. *RNA isolation and reverse transcription*

Total RNA was isolated using Total RNA Kit (A&A Biotechnology, Poland). The frozen tissues were homogenized with a vibratory mill Retsch MM-2 (Retsch, Germany). The purity and concentration of total RNA were determined by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Samples with A260/A280 ratio between 1.8 and 2.0 and RNA integrity number (RIN) between 9.1 and 10 were used in the experiment.

One microgram of RNA was treated with DNase and subjected to reverse transcription for 60 min at 42 °C in 20 μl of reaction mixture containing RT-buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT; Promega, Madison, USA), 10 mM of each dNTP, 500 ng of anchored oligo (dT)23 primers, and 200 U of reverse transcriptase (Promega, Madison, USA). The reaction was terminated by heating for 10 min at 70 °C.

2.3. *Quantitative Real-time PCR*

Thirteen housekeeping genes were selected to analyze the gene expression stability (Table 1). These genes were most often used as housekeeping genes in other species [19,20,4,21]. The *SUZ12*, *C2orf29* and *ZNF131* showed the highest stability of the expression in bovine endometrium [21]. Primers were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) with optimal T_m set to 60 °C for each primer. Primers were positioned in different exons to avoid the amplification of genomic DNA.

Real-time PCR was performed using Fast SYBR Green PCR Master Mix (Applied Biosystems) in the Applied Biosystems 7900 Real-time PCR System (Applied Biosystems). Reaction samples in total volume of 20 μl included cDNA (100 ng for each primer), 10 μl of Fast SYBR Green PCR Master Mix, and 0.2 mM of both PCR primers for the tested gene. The Real-time PCR was performed with an initial denaturation (20 s at 95 °C), followed by 40 cycles of denaturation (1 s at 95 °C), annealing and extension (20 s at 60 °C). cDNA in the reaction mixture was replaced by nuclease free water (non-template control sample). Melting curve was acquired to ensure that a single product was amplified in each reaction.

2.4. *Data analysis*

Real time-PCR data were analyzed using SDS 2.3 Software (Applied Biosystem). The variations between the cycle threshold (C_t) and the coefficient of variation (CV) for each housekeeping gene were plotted. Real-time PCR amplification efficiencies were determined by LinReg software, which calculates efficiencies using linear regression analysis of the log-linear region present within the lower region of the amplification profile [22]. The mean C_t values for each housekeeping gene were transformed using the comparative ΔC_t method with the following equation: $Q = E^{-\Delta C_t}$. The Q value is a relative quantity of gene expression, the E value is the reaction efficiency and the $\Delta C_t = C_t$ sample $- C_t$ min (where C_t sample is the C_t value for each housekeeping gene and C_t min is the minimal C_t value over a range of samples for a given gene). Relative gene expression data were analyzed using the GeNorm and NormFinder Microsoft Excel applets.

The GeNorm software determines the gene expression stability factor (M) which is an average pairwise variation for the tested gene compared to all other houskeeping genes. The exclusion of the gene with the highest M value allows the ranking of the tested genes according to their expression stability. This program takes into account the intra-group variability. Therefore, all CLs from the estrous cycle and pregnancy were included in one experimental group. The GeNorm calculates also the lowest number of housekeeping genes needed for the optimal determination of gene expression. This is achieved by determining the pairwise variation (V_n/V_{n+1}) between sequential normalization factors (NF) (NF_n and NF_{n+1}). A large variation indicates that the added gene has a significant effect and should be included for normalization. The value of 0.15 for the pairwise variation was established, below which the inclusion of additional reference genes is not necessary [23]. The NormFinder software estimates not only the overall variation of the candidate normalization gene but