ORIGINAL ARTICLE

# Development of real-time PCR assays in the study of gonadotropin subunits, follistatin and prolactin genes expression in the porcine anterior pituitary during the preovulatory period

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**Abstract**

**OBJECTIVES:** Neural control of the anterior pituitary function consists of the interplay of neuropeptides action, gonadal steroid hormones and many other factors. The physiological effect of this regulatory action is the release and synthesis of protein hormones in the precise time and quantity. The main factor responsible for the gonadotropins release and synthesis is the gonadotropin-releasing hormone (GnRH). We must still study the modulation of the synthesis of the gonadotropins subunits – LHβ, FSHβ and α subunit by different forms of GnRH and by its analogs, in order to better understand the regulation of gonadotropin release and synthesis. **THE AIM** of this study was to develop real-time PCR assays of five candidate reference genes for normalization purposes in order to quantify target transcripts in anterior pituitary cells during the preovulatory period. Moreover, we focused on the influence of GnRH receptor antagonist (antide) treatment on mRNA expression levels of GPα, LHβ, FSHβ, FST(follistatin) and PRL(prolactin) genes in these cells. **MATERIAL AND METHODS**: Anterior pituitary cells were obtained from pituitary glands of four mature pigs at the preovulatory phase. Cells were incubated with or without antide and relative mRNA level of target genes was measured using the Applied Biosystems 7500 Real Time System. For an exact comparison of mRNA quantity, the stability of five reference genes, ACTB, B2M, GAPDH, RPL1, and TOP2B was evaluated to choose the most appropriate reference gene for qRT-PCR normalization in the pituitary cells. Expression stability of reference genes was calculated using the geNorm application. The developed method of PCR assay was applied to study gene expression in pig pituitary cells in short culture. **RESULTS**: The most stably expressed genes in the pituitary cells

were GAPDH and TOP2B. The expression of ACTB, B2M and RPL1 appeared to be highly unstable. After normalization to the GAPDH/TOP2B, results showed that the mRNA expression of the FSHβ gene was highest in comparison with LHβ, GPα, FST and PRL genes ($p<0.005$). Pre-treatment of cells by the antide resulted in lower mRNA expression of these genes, while FSHβ mRNA had a significantly lower expression ($p<0.05$) in comparison with control.

**CONCLUSIONS:** Real-time PCR analysis of the expression of LHβ, FSHβ, α subunit, follistatin and prolactin genes in porcine anterior pituitary cells during the preovulatory period is suitable for the study of modulatory action of metal complexes with GnRH on the expression of these genes.

## INTRODUCTION

Successful animal reproduction requires synchronization of complex cellular mechanisms which occur at the level of hypothalamus, pituitary gland and gonads. A key regulatory role in these processes is played by neuropeptide GnRH which is synthesized in specialized neurons within the preoptic area of the hypothalamus and released in a pulsatile manner from the nerve endings of median eminence to portal circulation of the pituitary gland. GnRH exerts a physiological effect by binding to specific receptors on pituitary gonadotrophic cells which consequently stimulates the release and biosynthesis of both gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are dimeric glycoproteins in which the common α subunit is bound with the specific β subunit [17]. The α subunit gene expression in the anterior pituitary is regulated in a less specific way than LHβ and FSHβ subunit genes [22].

Among several trace elements important to maintain living systems, copper has been recognized for its involvement in both GnRH synthesis and release as well gonadotropin release. Indeed, studies revealed that copper ions complexed with histidine significantly promoted GnRH release from both isolated granules and hypothalamic explants [6, 1, 10, 9, 11]. Copper ions have been shown to stimulate both basal and GnRH-stimulated LH release from pituitary cells of immature female rats in vitro [8]. It has been suggested that copper ions modify the spatial structure of the GnRH receptor and stimulate mobilization of ionic calcium resulting in the release of gonadotropins from granules and cells to the extra-cellular space [18, 26].

Therefore, research on the modulation of complexes of GnRH with copper and other metals on the intracellular pathway(s) leading to the release and biosynthesis of gonadotropins and other pituitary hormones may introduce new tools for research and potential therapeutic application. Ratcliffe et al. [30] reported that the GnRH antagonist molecule conjugated with the progesterone was able to bind with plasma proteins and exhibited both GnRH and progesterone properties with increased potency and this new antagonist's complex served as a therapeutic agent for hormone-dependent diseases and as a contraceptive. It cannot be excluded that metal-GnRH complexes could also be useful in this field. Recently we have reported that in comparison to non-complexed peptide, Cu-GnRH molecule is more resistant to enzymatic degradation, both in hypothalamic and pituitary tissue [25]. Copper and nickel salts had no effect on the LH release from the pig pituitary cells in vitro while the complex of copper with GnRH (Cu-GnRH) exhibited potent stimulatory activity [24, 26]. In contrast to GnRH which operates mainly via $IP_3$ /PKC system, Cu-GnRH as well as Co-GnRH have been shown to activate the cAMP/PKA signaling pathway in porcine pituitary cells with minimal effect on $IP_3$ synthesis [4, 23]. The effect of the metal complexes with GnRH on gonadotropins and other pituitary hormones genes expression have been not explored so far and the present study is the first approach to study this problem.

Our preliminary results showed that both the GnRH and Cu-GnRH significantly stimulated follistatin gene expression in the anterior pituitary cells after 24 hrs of incubation [31, 3].

Quantification of the mRNA of genes whose transcription is stimulated by GnRH can be a powerful tool for the investigation of genes' regulatory networks and biochemical pathways involved in mammalian reproduction. Pig cDNA microarrays allow for high-throughput gene expression analysis in porcine tissues but real-time PCR is still the most sensitive method for quantifying mRNA transcripts and is suitable when only a small number of cells is available [15, 7]. However, data processing can affect the interpretation of real-time PCR results. Several critical steps need to be optimized such as standard curve of serial dilutions, PCR efficiency and normalization factor based on appropriate reference genes [28, 20]. In order to assess the amount and quality of nucleic acids, the reaction inhibitors of this internal control should not vary in the anterior pituitary cells under experimental conditions and in response to GnRH antagonist treatment.

The purpose of this study was to develop real-time PCR assays of five commonly used housekeeping genes. β-actin (ACTB) is a cytoskeletal structural protein. β-2-microglobulin (B2M) forms the β-chain of major hisocompatibility complex class I molecule. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is known for its role in glycolysis and gluconeogenesis. Ribosomal protein L1 (RPL1) is a structural component of the large 60S ribosomal subunit. Finally, topoisomerase 2-β (TOP2B) which is responsible for the control of topological states of DNA for the accurate quantification of target genes: GPα (α subunit of gonadotropins) LHβ, FSHβ, FST( follistatin) and PRL (prolactin) in the porcine anterior pituitary. To avoid co-expression, candi-

date reference genes belonging to different functional classes were selected.

The aim of this study was to develop real-time PCR assays of five candidate reference genes for normalization purposes to properly quantify target transcripts in pig anterior pituitary cells during the preovulatory period. Moreover, we focused on the influence of GnRH receptor antagonist (antide) treatment on mRNA expression levels of GPα, LHβ, FSHβ, FST and PRL genes in these cells.

## MATERIALS AND METHODS

*Pituitary cell culture*

Pituitary glands were collected from mature pigs (n=4) in the preovulatory phase of the estrous cycle. Anterior pituitary cells were obtained by sequential digestions using 0.25% trypsin solution (BIOMED, Lublin, Poland). After a final washing, cells were counted and re-suspended in McCoy's 5A medium (Sigma-Aldrich, St. Louis, USA) containing 10% horse serum, 2.5% fetal calf serum (BIOMED, Lublin, Poland) and antibiotics (penicillin/streptomycin; Sigma-Aldrich) at a density of $5 \times 10^5$ cells/ml. The cells were transferred to 24-well plates and cultured for 72 h at 37 °C. After attachment was completed, the cells were washed twice and pre-incubated with 0 or $10^{-7}$ M of antide (Sigma, St Louis, USA) for 30 min. Then, pituitary cells were washed with sterile PBS, and 0.2 ml of fenozol (A&A Biotechnology, Gdynia, Poland) was added to each well. Cells from the same treatment were pooled together and stored at –80 °C until RNA isolation.

*Total RNA extraction and reverse transcription (RT-PCR)*

Total RNA from the pituitary cells was isolated using Total RNA Mini (A&A Biotechnology, Poland) according to the manufacturer's protocol. Quantity and quality of total RNA was estimated by Nanodrop (Nanodrop, Wilmington, USA) and Bioanalyzer (Agilent, Santa Clara, USA). Isolated RNA samples were treated with RNase-free DNase (Promega corp., Madison, USA) to remove any possible contaminating genomic DNA and then dissolved in diethylpyrocarbonate-treated water.

The reverse transcription PCR (RT-PCR) was performed using 40 µl reaction mix containing 1 µg RNA, 5 µl M-MLV Reverse Transcriptase 5X Reaction Buffer, 10 U of RNasin, 0.5 µg oligo $(dT)_{15}$ and 100 U of M-MLV Reverse Transcriptase (all from Promega corp., USA). RNA was denaturated at 71 °C for 5 min, chilled on ice for 5 min and then the reaction mix was added. The mixture was then incubated at 42°C for 60 min. To deactivate the reverse transcriptase, the reaction mixture was heated and incubated at 95 °C for 3 min. The RT-PCR product was stored at –20 °C for further use.

*Primers and real-time PCR quantification*

Five genes were selected: ACTB, GAPDH, B2M, TOP2B and RPL1 belonging to different functional classes, frequently used as references in real-time qPCR gene expression experiments. Primers for GAPDH, B2M, TBP and RPL1 were used from Erkens et al. [13]. Primers for ACTB, TOP2B, as well as for target pituitary hormones genes: LHβ, FSHβ, GPα, PRL and FST were designed using the Primer 5 software (Whitehead Institute/Massachusetts Institute of Technology, Boston, USA) and

**Table 1**. Primer sequences for evaluated reference genes and investigated target genes.

| Gene | Primer sequence | Amplicon length (bp) | Ta (°C) | Accession number |
|---|---|---|---|---|
| ACTB | GGACTTCGAGCAGGAGATGG<br>GCACCGTGTTGGCGTAGAGG | 130 | 59 | DQ178122 |
| GAPDH | ACTCACTCTTCTACCTTTGATGCT<br>TGTTGCTGTAGCCAAATTCA | 100 | 57 | DQ178124 |
| B2M | AAACGGAAAGCCAAATTACC<br>ATCCACAGCGTTAGGAGTGA | 178 | 60 | DQ178123 |
| TOP2B | AACTGGATGATGCTAATGATGCT<br>TGGAAAAACTCCGTATCTGTCTC | 137 | 60 | AF222921 |
| RPL1 | AGTTAAAGTACCTGGCCTTCCT<br>TGGCCTCTCTTGGTCTTG | 136 | 59 | DQ178127 |
| LHβ | CAGGGACTGCTGTTGTGGC<br>AGGCGGATGGAGGCAAA | 254 | 60 | NM214080 |
| FSHβ | ACAAGGCAGCCGATCACAGTTT<br>CGGGTGTAGCAATAGCCAGCA | 229 | 60 | U78049 |
| GPα | TGCAGCGAGGTCCAAGAAGA<br>CCACTCTGGCATTTCCCATTAC | 113 | 60 | NM214446 |
| FST | CCGAATGAACAAGAAGAACAAACC<br>TGGAGCTGCCTGGACAGAAAA | 215 | 60 | NM001003662 |
| PRL | GCAGAGGGTTCATTACCAAGGC<br>AGGTGATACAGCGGGTCATTCC | 154 | 60 | NM213926 |

porcine GenBank sequences. Primer sequences and GenBank accession numbers are given in Table 1. Primers were designed to produce amplicons spanning two exons.

The PCR amplification was performed in two independent runs using a 7500 ABI PRISM apparatus (Applied Biosystems, Foster City, USA) using 96-well optical plates with a SYBR GREEN PCR Master Mix technique (Applied Biosystems, USA). A PCR mix (25 µl) was prepared to give the indicated final concentrations: 11.1 µl water, 0.2 µl primers (forward and reverse; 10 µM), 1 µl (100 ng) cDNA, and 12.5 µl SYBR GREEN PCR Master Mix. The following amplification program was used: 10 min of denaturation at 95 °C, 40 cycles of 4-segment amplification with 15 s at 95 °C (for denaturation), 30 s at 58–60 °C (for annealing), and 40 s at 72 °C for elongation. Annealing temperatures were optimized to individual genes and primers. A dissociation step was added to ensure that the desired amplicon was detected. The dissociation eliminated a non-specific fluorescence signal and ensured the accurate quantification of the desired product. Subsequently, a melting step was performed consisting of 2 s at 95 °C, 5 s at 58 °C and slow heating with a rate of 0.1 °C per second up to 95 °C, with continuous fluorescence measurement, and finally followed by cooling down to 35 °C. Standard curves were made for calculating the amplification efficiency during real-time PCR. Five dilutions of each cDNA (0x, 5x, 25x, 125x, 625x) were primed separately for each reference gene.

*Data processing and statistical analysis*

Reference gene validation analysis was performed using qRT-PCR method as described above. Gene expression variation was calculated for individual reference genes based on cycle threshold (Ct) value and real-time PCR efficiency (E). Real-time E was calculated from the given slopes in the 7500 Real Time PCR System software according to the equation: ($E = 10^{-(1/b)} - 1$; b = regression coefficient). The Ct value is defined as the number of cycles needed for the fluorescence signal to reach a specific threshold level of detection and is inversely correlated with the amount of template cDNA present in the reaction [36]. Ct and E values were entered into geNorm application, which ranks genes based on M-value in which the gene with the most stable expression has the lowest M-value. If variation is observed in the expression ratios it means that genes are not constantly expressed, which results in increased variation of ratios and decreased expression stability. M-values were first calculated using the whole set of genes, then the gene with highest M-value (the least stable gene) was excluded from the set and M-values were calculated for the remaining genes [35]. For subsequent normalization of qRT-PCR data, two most stably expressed reference genes were used. Relative expression level of target genes based on Ct and E values in comparison to normalization factor (NF) was calculated for individual genes using the mathematical model described by Pfaffl [28]. NF was obtained from geometric mean of the raw expression data of the two most stably expressed reference genes.

One-way analysis of variance (ANOVA) was used to test for differences among groups. ANOVA was followed by Tukey, Scheffe and Bonferroni post-hoc tests. T-tests were performed to check for differences between groups (control and antide) for individual genes. All statistical analyses were conducted using the STATISTICA base 7.1 software (Statsoft Inc., Tulsa, USA). Data on diagrams are reported as the mean ± standard error of the mean. Differences between groups were considered significant at $p<0.05$ and $p<0.005$.

## RESULTS

*Reference gene selection*

Five reference genes were amplified in examined cDNA samples and Ct values were detected for all genes. All real-time PCR assays produced a single peak in the melting curve. Single band products of appropriate size were obtained with agarose gel electrophoresis (not shown). Reaction efficiencies, measured using serial dilution of cDNA, were between 88–95%. According to the results obtained with the geNorm application, the most stably expressed genes in anterior pituitary cells samples are GAPDH and TOP2B. Of note, ACTB, B2M and RPL1 proved to be highly unstable in our anterior pituitary samples. The M-values were used to rank the genes on the basis of their stability from least to greatest (Table 2).

Results of the LHβ, FSHβ, GPα, FST and PRL mRNA expression in the pig anterior pituitary cells prepared from pituitaries taken from the animals in the preovulatory period are shown in Figure 1 and expressed in the arbitrary units with standard errors (SEM). The mRNA expression of FSHβ gene was significantly elevated in the control group ($p<0.005$) in comparison to the antide treated group and with LHβ, GPα, PRL and FST genes.

Although the antide pretreatment lowered the expression of LHβ, FSHβ, GPα, FST and PRL genes in anterior pituitary cells collected from the preovulatory phase of the estrous cycle, the significant decline ($p<0.05$) was observed only for FSHβ mRNA expression (6.43+0.95 vs. 3.29+1.09 ).

## DISCUSSION

So far there has been no information concerning the expression of reference genes and real-time PCR data normalization in pig anterior pituitary cells. In this study five reference real-time PCR assays were designed to measure the pituitary mRNA level of five anterior pituitary expressed genes related to reproduction. Since

**Table 2**. Expression stability of housekeeping genes analyzed with the geNorm program. Genes with the lowest M-values have the most stable expression.

| Average mRNA expression stability (M-values) | |
|---|---|
| **least stable genes** | **most stable genes** |
| B2M (1.8); RPL1 (1.5); ACTB (1.13) | GAPDH/TOP2B (0.79) |

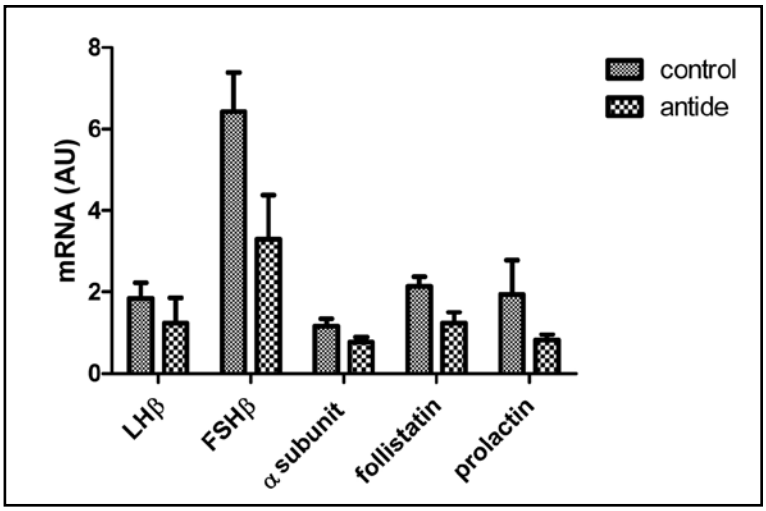


**Figure 1.** Expression of LHβ, FSHβ, GPα, FST and PRL in the pig anterior pituitary cells collected from the animals at the preovulatory stage of the estrous cycle. The effect of antide ($10^{-7}$ M) treatment was compared with the control cells (without antide). Data are reported as the mean ± SEM. Significance: *P<0,05 of FSHβ antide group as compared to control FSHβ (T-test), **P<0,005 as compared to other genes in control group (ANOVA).

the choice of a proper reference gene is a fundamental problem for the normalization of real-time qPCR data and in microarray experiments [2, 8, 37], several potential reference genes that pass the same steps of analysis as the gene(s) to be quantified [29] should have been tested. As shown in the present study, both TOP2B and GAPDH, commonly used reference gene, were mostly stable among the set of tested reference genes. Consequently, they appeared to be the best control genes used in the pig anterior pituitary hormone gene study. Recently it has been shown that the expression of the TOP2B gene was highly stable also in such pig tissues such as muscle, kidney and liver [13, 34]. In contrast, RPL1, B2M as well as ACTB genes were less stably expressed in our experimental conditions. It is now well recognized [5] that B2M is an essential subunit for the expression of MHC class I and nonclassical MHC molecules [5]. Less stable expression of B2M gene observed in our research might be due to altered cellular expression of MHC class I and B2M in pigs. Although the RPL1 encode component of the 60S ribosomal subunit which is involved in protein biosynthesis [32] and rRNA molecule was proposed as an appropriate reference gene, the observed imbalance between cellular rRNA and mRNA fractions do not support the choice of this gene for q-RT-PCR gene(s) quantification [35; 27]. Moreover, gene expression results obtained in porcine anterior pituitary may be more reliable if they are normalized using the geometric means of multiple reference genes. Such a procedure was also recommended for other species [12, 21]. Our results demonstrate that the two reference genes TOP2B and GAPDH should be used as controls to validate gene expression data from the investigated porcine tissue.

In our study we cultured anterior pituitary cells obtained from pigs in the preovulatory phase of the cycle. Since after pituitary excision cells were prepared relatively quickly for culturing (5 hrs), the influence of the hormonal background characteristic for living organism could also be at least partially maintained in in vitro conditions. Hiatt and Schwartz [19] showed in vitro that the sensitivity to GnRH pulses were significantly greater in proestrous than in metestrous rat pituitaries as well as that preincubation with the GnRH receptor antagonist did not lower the basal secretion rate of LH at metestrous. Moreover, despite highly lowered basal LH release observed in dispersed rat pituitary cells, their GnRH responsiveness pattern still reflects the day of the cycle [14].

Preliminary results evidenced that both GnRH and Cu-GnRH exhibited the significant stimulatory effect on follistatin gene expression in the pig anterior pituitary cells [3, 31]. In the present experiment, treatment of pig pituitary cells with antide resulted in lower expression of the five studied genes. Antide inhibited the stimulatory action of GnRH on the LHβ expression in female rats [16] but stimulated the Cres gene expression 3-fold in normal male mouse anterior pituitary gonadotropes and exhibited a little effect in castrated animals [33]. Antide may be a helpful tool for the investigation of the complex mechanisms responsible for the regulation of different anterior pituitary gene expression observed after stimulation of metal-GnRH complexes

Results of the present study also provide new information on the HKG references to be used in gene expression experiments carried out on the porcine pituitary.

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