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Expression of Growth Hormone Gene in the Pituitary of Piglets Born to Gilts Injected with Pregnant Mare Serum Gonadotropin and human Chorionic Gonadotropin Prior to Mating

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Abstract

Injection of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) are expected to improve endogenous secretions of pregnant hormones that will improve gestation quality that will be expressed in the improved growth performance of the piglets born. Growth and development are controlled by growth hormone (GH) gene. This research was conducted to study the expression of GH gene in the piglets born to gilts injected with gonadotropin prior to mating. The experimental gilts used were Landrace that were divided into two groups: 1) gilts injected with physiological NaCl 0.90% solution as control, and 2) gilts injected with PMSG and hCG to increase endogenous secretions of pregnant hormones. The experimental gilts were maintained during pregnancy until parturition.

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At the age of 100 days postpartum, 8 piglets (4 piglets born to gilts injected with NaCl 0.90% as control and 4 piglets born to gilts injected with PMSG and hCG) were selected from the experimental piglets born in the same litter size of 8 and were euthanized for measurement of GH gene expression in the pituitary. Messenger ribonucleic acid (mRNA) was extracted from pituitary tissue to observe differences in GH gene expression of piglets using real time quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), with β-actin as a housekeeping gene. The results showed that piglets born to gilts injected with PMSG and hCG prior to mating had higher GH gene expression in the pituitary even though the increase was not statistically significant in the t-test analysis ($P>0.05$). It is concluded that the improved prenatal growth and postnatal growth of piglets born to gilts injected with gonadotropin was associated with the increased expression of growth hormone gene.

Keywords: Pregnant mare serum gonadotropin; human chorionic gonadotropin; gene expression; growth hormone gene; pituitary.

1. Introduction

In mammalian animals, the success of reproduction is determined by the quality of fertilized oocytes and the readiness of the uterus to accept the blastocyst for implantation and further growth and development of uterus and placenta to support the growth and development of embryo and fetus until parturition. The degree of uterine growth and development will affect the synthesis and production of nutrient, growth factors, and hormones that control and regulate embryonic growth and development [1, 2]. The growth and development of uterine glands are controlled mainly by the estrogen produced by the ovary during estrous cycle and progesterone produced by the corpus luteum and placenta during pregnancy.

Un-optimum progesterone synthesis and secretion during pregnancy will reduce the capacity of the uterine environment to support the growth and development of conceptus as indicated by the decreased conceptus elongation and low conception rate [3] and decreases the gene expression of endometrium and secretion of histotroph into the lumen of uterus [4]. Progesterone supplementation in the beginning of pregnancy improves the growth and development of embryo, conceptus elongation, interferon tau production [5], and embryonic survival [6]. Intra-vaginal supplementation of estradiol and progesterone improves growth and development of embryo [7] and placenta [8] that finally improves prenatal growth. Increased progesterone synthesis and secretion during pregnancy increased the success of pregnancy [9] through its effect on the improvement of embryo survival and growth and elongation of blastocyst [10]. The improved uterine environment will support the optimum growth and development of embryo and fetus until parturition to produce healthy and superior offspring.

In mammalian animals, it is proved that injection of the mother with gonadotropin prior to mating improves endogenous secretions of pregnant hormones during pregnancy. Improved endogenous secretion of pregnant hormones during pregnancy improve uterine, placental, embryo and fetal growth that finally improved birth weights and post-weaning weights of the offspring in sheep [11, 12], goat [13], and pigs [14, 15, 16, 17]. The improved post-natal growth performances of the offspring are also improved health performances as indicated by the significantly higher fitness and lower mortality rates.

Generally, un-optimum uterine and placental growth and development during pregnancy will modulate the endocrine status and the expression of genes related to growth and development [18] that further limits the growth and development embryo and fetus that eventually decrease birth weight and the fitness of the offspring with postnatal growth rate [19, 20]. Therefore, the improved growth performances of the offspring born to mother injected with gonadotropin prior to mating could be related to the improved expressions of genes related to the growth and health performances. One of the genes that control the growth and health performance is encoding growth hormone (GH) gene which plays a role in the secretion of GH [21]. The hypothalamus secretes growth hormone releasing hormone (GHRH), which stimulates the anterior pituitary of brain to produce GH [22]. The GH gene have an important role in reproduction, embryogenesis, lactation and other growth [23, 24] even in the post-natal growth performance [25]. The present experiment was conducted to measure the expression of growth hormone gene in the pituitary of the piglets born to control and gonadotropin-injected gilts prior to mating. This study used β-actin gene as a housekeeping gene for data normalization in the quantitative Reverse Transcription - Polymerase Chain Reaction (qRT-PCR) process. The expression of mRNA of GH genes in the pituitary tissue of piglet born to gilts injected with gonadotropin prior to mating has not been reported. The limitation of this study is the quality improvement efforts of gestation with gonadotropin injected sows prior to mating, than analyzed the expression of GH gene in piglets born to gilts. The results of this study are expected to acquire data on the GH gene expression in the pituitary of piglets born to gonadotropin injected sows prior to mating.

2. Materials and Methods

2.1. Piglets Production

Piglets used in the measurement of GH gene expression were obtained from the collection of piglets born to control and gonadotropin-treated sows. The gilts used in the experiment are Landrace progeny in pig breeding company of CV Wailan, Tomohon, North Sulawesi Province, Indonesia. Before mating, the estrus cycles of the experimental gilts were synchronized by injecting the gilts twice with prostaglandin $PGF_2\alpha$ (Lutalyse, Intervet, Netherlands) with 14 days interval. At the second prostaglandin injection, 16 gilts were injected with 0.9% physiological NaCl as control and 16 gilts were injected with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) (PG 600, Intervet, Netherlands) prior to mating at a dose of 600 IU per gilts. After estrus, the experimental gilts were mixed with selected boars for natural mating. During pregnancy and lactation, the experimental gilts were maintained and raised according to the management adopted in the company. The total number of piglets used in the measurement of GH gene expression were 8 piglets consisted of 4 piglets born to control gilts injected with NaCl 0.90% and 4 piglets born to sows injected with gonadotropin prior to mating. The piglets used for measurement of GH gene expression were selected from litter size of 8. The measurements were conducted at the age of 100 days post-partum. The experimental piglets were euthanized and the pituitary was isolated for measurement of the mRNA expression of GH gene.

2.2. Primer Design

The primers used in the experiment consist of gene of GH as a primer gene and gene of β-actin as housekeeping gene. The primer oligonucleotide was designed based on the gene sequences of GH and β-actin in GeneBank.

The primer sequences of GH gene (AY536527) is forward 5'-TGG TGT TTG CCT GCA CAG AC -3 'and reverse 5'-CGT CAT CAC TGC GCA AGT TT -3' with a size of 159 bp. The prime sequences of β-actin gene (DQ845171) is forward 5'-CAT CCT CCT GCG TCT GGA G -3 'and reverse 5'-CCA TCT CCT GCT AGT CGA CC -3' with a size of 161 bp.

2.3. mRNA Extraction

The extraction of mRNA was derived from pituitary tissues of experimental piglets. Around 1 g of isolated pituitary tissue was taken aseptically and stored in 1.5 ml eppendorf tubes containing RiboSaver RNA stabilization solution until the tissues were submerged, then the mixture was stored at a temperature of -4 °C until the time of analysis. The mRNA was extracted using GeneJet RNA Purification Kit method (Thermo Scientific, Lithuania, EU).

2.4. Quality Test of the extracted mRNA

The concentration and purity of the mRNA were analyzed using a spectrophotometer. The mRNA purity is correlated with the quality of mRNA that was determined by the level of protein contamination by dividing the value of the optical density at a wavelength of 260 nm (OD_{260}) with the value of the optical density at a wavelength of 280 nm (OD_{280}). If the obtained values ranges from 1.80-2.0 (260/280> 1.80), then the mRNA was categorized as pure [26].

2.5. Reverse transcription - Polymerase Chain Reaction (RT-PCR)

Reverse transcription in which the mRNA is transcribed back into cDNA by the enzyme of reverse transcriptase using a kit of Transcriptor Synthetis First Strand cDNA (Thermo Scientific, Lithuania, EU). The RNA templates used were RNA template of sample and the standard each with the volume of 2 mL. The results obtained were template in the form of cDNA samples and cDNA standard. The next stage was optimization and operation of qRT-PCR (Analytic Jena, AG qTower 4 channels, Germany). The optimization aimed to obtain a good standard for the RT-PCR results of the samples. The samples were distributed into the RT-PCR tube, and centrifuged horizontally on 25000 rpm for 10 seconds. The material consisted of 1 µl of cDNA sample, 4.9 µl H2O, 5 µl mastermix (CybrGreen), and 0.2 µl primer (forward and reverse). Furthermore, the operation of RT-PCR machine were conducted with the following conditions; 95°C for 5 min, 95°C for 10 seconds (denaturation), followed by 60°C for 20 seconds (annealing) and 72°C for 30 seconds (extension). The PCR process lasted for 39 cycles.

2.6. Data Analysis

The quantification of GH gene expression was calculated based on the approach of the relative number of targeted genes (GH) and housekeeping genes (β-actin), with comparative cycle threshold (C_T), $\Delta C_T = C_T\ GH - C_T\ \beta\ aktin$. The formula to calculate the delta C_T is $\Delta\Delta C_T = \Delta C_T\ treatment - \Delta C_T\ control$ [27]. The relative quantification of the expression between the target genes and controls gene were compared with the equation of $2^{-\Delta\Delta CT}$ [28]. The data on the mRNA expression of GH gene on pituitary of piglets was presented in the average

value ± standard error. The data were statistically analyzed using t-test with a significant level of α=5%. All data were processed using SPSS 20 computer software.

3. Result and Discussion

Before conducting the gene expression analysis, a measurement of the quality of mRNA was needed to assess whether mRNA was feasible to be used and it is not contaminated by the others. If the value of the ratio OD_{260}/OD_{280} on the measurement results using spectrophotometer was less than 1.80, then the mRNA was contaminated with protein. The results obtained in this study ranged from 2.03 (260/280>1.80) so the quality of mRNA was good for further analysis stage of gene expression using qRT-PCR machine.

3.1. The average value of CT Gene β-actin with the average of C_T Gen GH on qRT-PCR

The phenotype is controlled by genes, whether the gene will be expressed or in an inactive state (silent). The data of qRT-PCR analysis are presented in the form of graphs and quantification value in the form of DNA copy number after being calibrated with threshold value. The value of cycle threshold (C_T) of the qRT-PCR results showed that the gene analyzed was expressed in the piglet's pituitary tissue. The results showed that the average of gene C_T of GH target was higher than the average C_T β-actin (Table 1). Actin is an essential protein for eukaryotic cells. This protein has important roles in forming tissues that provide mechanical support for cells, determine cell shape, cell movement, and cell division. B-actin gene had a stable level of expression in various tissues at all stages of development. The nature of such gene that makes gene β-actin was used as internal controls in the analysis of gene expression by qRT-PCR method [29, 30].

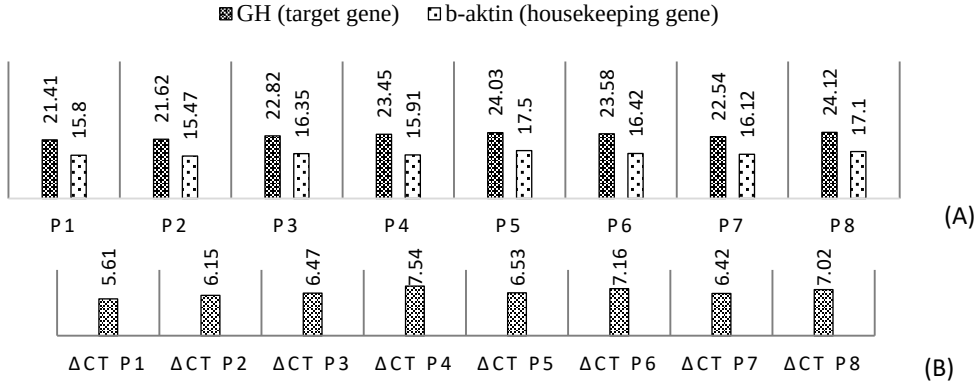

Figure 1: (A) Comparison of C_T GH genes (target) with β-actin gene (housekeeping gene), P1 to P4 were the C_T GH genes of piglets born to gilts injected with physiological NaCl 0.90%, P5 to P8 were the expression of C_T GH genes of piglets born to gilts injected with PMSG-hCG prior to mating. (B) The results of the C_T GH genes reduction (target) with β-actin gene.

3.2. Effect of injection of gilts with gonadotropin prior to mating on ΔC_T of GH gene in the pituitary of the experimental piglets

GH genes from the pituitary tissues of piglets born to gilts injected with PMSG and hCG prior to mating had ΔC_T values that were higher than those found piglets born to control gilts injected with physiological NaCl (Table 1). The ΔC_T values of the pituitary quantitatively showed that injection of the gilts with PMSG and hCG prior to mating enhance GH gene expression. However, the difference is not statistically significant (P> 0.05). These results were probably caused by the role of hypothalamus in controlling GH secretion. The pituitary gland secretes nine hormones that regulate the homeostasis of the body, including the pituitary internal environment and tends to maintain a constant and stable condition.

Table 1: Expression of mRNA of GH gene (ΔCT) (mean ± SE) in the pituitary of piglets born to gilts injected with NaCl 0.9% (control) and PMSG-hCG prior to mating

Parameter	Piglet born to gilts injected with		Average
	NaCl 0.9%	PMSG – hCG	
ΔC_T pituitary	6.44 ± 0.407	6.78 ± 0.181	6.61 ± 0.216

The increase in the expression of GH gene in the piglets born to PMSG-hCG injected gilts prior to mating in this study was the effect of improving uterine environment during pregnancy due to the increased endogenous secretions of pregnant hormones during pregnancy. Optimum uterine environment during pregnancy will optimize the growth and development of embryo and fetus until parturition as an indicator of optimum genetic expression. The injection of PMSG and hCG improves the pregnancy condition of the sow to produce piglets with good quality of growth, as the result of PMSG and hCG which improve the uterine environment by increasing the secretion of pregnant hormones (estrogen, progesterone and placental lactogen) [14]. The conditions in the uterus not only affect the health of piglets in newborn condition, but also affect their life qualities during adult age [31].

3.3. Relative quantification of mRNA expression of GH gene in the pituitary of experimental piglets using Schmittgen and Livak methods ($2^{-\Delta\Delta CT}$)

After ΔC_T values of the pituitary piglets were obtained, then the relative quantification of gene expression were calculated using Schmittgen and Livak methods. Relative quantification of physiological NaCl injection as a control was compared to PMSG-hCG injection prior to mating. The relative comparison of gene expression of pituitary GH ($2^{-\Delta\Delta CT}$) is 1.266 (Figure 2) in piglets born to gilts injected with PMSG-hCG prior to mating, and 1.000 in piglets born to gilts injected with NaCl 0.90% as control.

The GH relative quantification of gene expression is seen from the GH gene expression level compared to β-actin (housekeeping gene) using the $2^{-\Delta\Delta CT}$ formula. The results of this study is similar to the results reported previously [32] that the level of gene expression in pituitary GH gene of Landrace pigs at the age of 35 to 125 days is in the range of 1.100 to 0.800 where the value of GH decreases with the increase in the age of the experimental animals.

The results showed that the pituitary of piglets born to gilts injected with PMSG and hCG prior to mating had higher expression of GH gene compared with those injected with physiological NaCl as a control. Injection of the gilts and sows with PMSG and hCG prior to mating will increase the endogenous secretion of pregnant hormones that will optimize the uterine environment during pregnancy through the improvement of nutrient, oxygen, and biological compounds availability for supporting optimum growth of embryo and fetus until parturition [33, 34]. The improved uterine environment during pregnancy will optimize the programming of fetal growth and development that could involve optimum gene expression [2, 35, 36, 37]. Our previous results clearly showed that injection of the mother with gonadotropin prior to mating improve endogenous secretion of pregnant hormones that improve prenatal growth as were indicated by the higher birth weight and better postnatal growth in sheep [11, 12], goat [13], and pigs [14, 15, 16, 17].

Thus, this study proves that the GH gene expression in piglets can be improved by improving endogenous secretion of pregnant hormones during pregnancy by injection of the sows with gonadotropin prior to mating. Further research is required to test whether the improved gene expression will be inherited to the further generation of offspring.

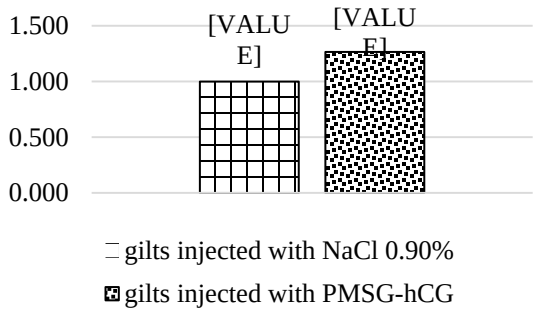

Figure 2: Relative quantification of GH gene expression ($2^{-\Delta\Delta Ct}$) in the pituitary of experimental piglets born to gilts injected with NaCl 0.90% and PMSG-hCG prior to mating

4. Conclusion

Improved prenatal growth and birth weights of piglets born to gilts injected with PMSG and hCG prior to mating were associated with the increased GH gene expression in the pituitary piglets. Relative quantification of GH gene expression in the pituitary of piglets born to sows injected with PMSG and hCG shows a higher value of 1.266.

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