Transcriptome analysis of the effect of pyrroloquinoline quinone disodium (PQQ·Na₂) on reproductive performance in sows during gestation and lactation

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Abstract

Background: Pyrroloquinoline quinone (PQQ), which is a water soluble, thermo-stable triglyceride-quinone, is widely distributed in nature and characterized as a mammalian vitamin-like redox cofactor. The objective of this study was to investigate the effects of pyrroloquinoline quinone disodium (PQQ·Na₂) on reproductive performance in sows.

Results: Dietary supplementation with PQQ·Na₂ significantly increased the total number of piglets born, the number of piglets born alive and the born alive litter weight. It also increased the antioxidant status in the placenta, plasma and milk. The concentration of NO was significantly increased in the plasma and placenta. RNA-seq analysis showed that 462 unigenes were differentially expressed between the control (Con) treatment and PQQ treatment groups. Among these unigenes, 199 were upregulated, while 263 unigenes were downregulated. The assigned functions of the unigenes covered a broad range of GO categories. Reproduction (27, 7.03%) and the reproduction process (27, 7.03%) were assigned to the biological process category. By matching DEGs to the KEGG database, we identified 29 pathways.

Conclusions: In conclusion, dietary supplementation with PQQ·Na₂ in gestating and lactating sows had positive effects on their reproductive performance.

Keywords: Oxidative stress, Pyrroloquinoline quinone, Pyrroloquinoline quinone disodium, Reproductive performance, RNA-seq, Sow

Background

Sow reproductive performance affects the production level and provides an economic benefit to the pig industry. Litter size is an important reproductive trait and a critical indicator of sow reproduction performance [1]. Maintaining optimal reproductive and litter performance is essential for meeting economic targets in commercial pig production [2]. Increasing litter size has long been a goal of pig breeders and producers; prolificacy is of great interest to the pig industry [3]. Maternal nutrition has substantial implications for fetal health. Enhancing reproductive performance through nutrition and management strategies in gestation and lactation sows have been of research interest for several decades [4]. The placental tissue is the only site for contact between the fetus and the mother during pregnancy; thus, the tissue is closely related to the health and development of the fetus [5]. Maternal conditions have been demonstrated to affect the placental morphology, blood flow, fetomaternal exchanges, and endocrine function [6]. In addition, the placenta plays a pivotal role in maternal nutrient supply and metabolic waste removal, protection against bacterial and viral infections, and production of hormones supporting pregnancy [7].

Pyrroloquinoline quinone (PQQ), which is a water soluble, thermo-stable triglyceride-quinone [8]. Initially identified as a novel cofactor of various bacterial dehydrogenases [9], PQQ is an essential animal nutrient.
PQQ-deficient animals display a variety of illnesses [10]. PQQ has attracted considerable attention, as it is important for mammalian growth, development, reproduction and immune function [11]. PQQ is an effective antioxidant, protecting mitochondria against oxidative stress-induced lipid peroxidation, protein carbonyl formation and inactivation of the mitochondrial respiratory chain [12]. Although PQQ is not biosynthesized in mammals, trace amounts of PQQ have been found in human and rat tissues at picomolar to nanomolar levels, and an especially large amount has been found in human milk [13].

Because of its versatile functions, PQQ disodium (PQQ·Na₂) salt has been authorized in Canada as a Natural Health Product, providing 20 mg PQQ·Na₂ salt per day as an antioxidant for the maintenance of good health [14]. On August 13, 2018, the European Commission issued regulations (EU) 2018/1122 approving pyrroquinoline sodium salt as a new type of food. The European commission defines pyrroquinoline sodium as a new dietary supplement. Although PQQ has a positive effect on reproductive performance, its mechanism is not clear. Therefore, in this study, we used the Illumina HiSeqTM2500 platform to perform a large-scale transcriptome analysis of the placenta of sows. The next-generation RNA sequencing (RNA-seq) is powerful for gene identification, comparative gene expression analysis and investigation of the functional complexity of the transcriptome [16]. In recent years, an RNA-seq approach has been widely used in animals for novel gene identification and differentially expressed gene (DEGs) analysis, because it is high throughput, low cost, covers a multitude of low abundance genes, and has high sensitivity. Although PQQ has gained interest in medicine and health-related research in recent decades, the usefulness of PQQ has not yet been fully demonstrated in animal agriculture, especially in the pig industry. In addition, there are no published data on the effects of PQQ on reproductive performance in sows. Therefore, the objective of this study was to test the efficacy of dietary PQQ·Na₂ supplementation on reproductive performance. We also analyzed the RNA-seq of placentas, which revealed the genes that may be involved in placental development and function, thus playing a role in determining litter size. Furthermore, the data collected were used to establish the relationship between PQQ and the placenta of sows, as well as reproductive performance. We hope the results of this study could lay a foundation for the further study of PQQ in the pig industry.

Methods

Animals and management

A total of 40 crossbred (Landrace×Large White crossed with Duroc boar) multiparity gestation sows with an average parity of 4.3 were used in the study. Forty sows were allotted to 2 dietary treatments after breeding. One group was the control sows, which were fed a corn-soybean meal control diet (Con treatment, n = 20), and the other group was the treatment sows, fed a control diet with 20 mg/kg PQQ·Na₂ after breeding and through gestation (PQQ treatment, n = 20). The PQQ·Na₂ (purity, ≥ 98%) was synthesized by chemical reactions. It was diluted with corn starch to a concentration of 1 g/kg mixture before being mixed into the diet. Based on the known range of PQQ in foods [17], we inferred that the concentration of PQQ in the basal diet was less than 0.01 mg/kg. The sows were kept in single crates (0.6 m × 2.0 m) from insemination to day 110 of gestation. On d 110 of gestation, sows were transported to the farrowing facility, where they were placed in individual farrowing crates (2.4 m × 2.4 m). Each crate had steel mesh floors with a heat lamp for newborn pigs. The crates were mounted over a solid concrete floor, and manure was removed manually each day. The farrowing room temperature was maintained at approximately 18 to 20 °C. Births were watched, but the observers interfered as little as possible in the farrowing process. The protocols used in this experiment were approved by the Northeast Agricultural University Institutional Animal Care and Use Committee. All animal experimental diets (Table 1) were formulated to meet or exceed the recommended nutrient requirements of the NRC (2012). From d 1 of gestation until d 90 of gestation, all sows were fed 2.5 kg of the gestation diet daily. From d 91 of gestation, all sows were fed 4.5 kg of the gestation diet daily. The amounts of parturition feed provided to each sow at d 112, 113 and 114 of gestation were 2.0, 1.5 and 1.0 kg, respectively.

Sample and data collection

Blood was collected from the ear vein of a random subset of sows (n = 8 per treatment) at d 90 of gestation and d 21 of lactation. The blood was centrifuged at 3000×g for 15 min to obtain the plasma, and the plasma was stored at −20 °C until analysis. Eight sows per treatment were randomly selected and marked for milk sample collection during lactation. Colostrum was collected within 6 h of farrowing (d 0 of lactation). Approximately 30 to 50 mL of milk was collected from all functional mammary glands using a mechanical milk pump after the injection of 1 mL oxytocin. The samples were immediately stored at −20 °C until analysis. Placenta allantochorion tissue samples were collected immediately during parturition to preserve RNA stability for mRNA...
Table 1 Composition and nutrient levels of diets

| Item                        | Gestation |
|-----------------------------|-----------|
| Ingredients, %              |           |
| Corn                        | 67.5      |
| Soybean meal                | 16        |
| Wheat bran                  | 13.5      |
| Dicalcium phosphate         | 1         |
| Limestone                   | 1.1       |
| Salt                        | 0.4       |
| Premix<sup>a</sup>          | 0.5       |
| Nutritional composition<sup>b</sup>, % |     |
| Net energy, MJ/kg           | 9.62      |
| Crude protein (CP)          | 15.58     |
| Calcium                     | 0.71      |
| Total phosphorus            | 0.60      |
| Available phosphorus        | 0.31      |
| SID Lysine                  | 0.54      |

<sup>a</sup>The premix provides following for per kg diet: vitamin A, 8000 U; vitamin D<sub>3</sub>, 2000 U; vitamin E, 50 U; vitamin K<sub>3</sub>, 1.5 mg; vitamin B<sub>1</sub>, 1.6 mg; vitamin B<sub>2</sub>, 1.5 mg; vitamin B<sub>6</sub>, 15 μg; niacin, 20 mg; D-pantothenic acid, 15 mg; Zn (ZnO), 100 mg; Fe (FeSO<sub>4</sub>·7H<sub>2</sub>O), 80 mg; Cu (CuSO<sub>4</sub>·5H<sub>2</sub>O), 20 mg; Mn (MnSO<sub>4</sub>·H<sub>2</sub>O), 25 mg; I (KI), 0.3 mg; Se (NaSeO<sub>3</sub>·5H<sub>2</sub>O), 0.2 mg

<sup>b</sup>Nutrient levels were calculated values

Analysis. A section of samples was stored at –20 °C, and another section was snap-frozen in liquid nitrogen for further analysis. Sow back-fat thickness was measured at d 0 and 90 of gestation, within 24 h of farrowing (d 0) and d 21 of lactation (n = 8 per treatment). Back-fat thickness was measured at the P2 position (left side of the 10<sup>th</sup> rib and 6 cm lateral to the spine) by digital B-ultrasonography (Kaixin, Xuzhou, China). At farrowing, the number of piglets born, litter birth weight and individual birth weights were recorded.

Evaluation of antioxidant enzyme activity and the concentration of nitric oxide

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) enzyme activities in the milk, plasma and placenta were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with our previous study. The results of the measurements were expressed as U/mL in plasma and milk and as U/mg protein in placenta. Lipid peroxidation in the plasma, milk and placenta was determined by measuring the amounts of malondialdehyde (MDA) through the thiobarbituric acid method using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The results of the measurements were expressed as nmol/mL in plasma and as nmol/mg protein in placenta. Nitric oxide (NO) and inducible NOS (iNOS) in the plasma and placenta of sows were determined using assay kits obtained from Jiaocheng Biochemistry (Nanjing, China). The colostrum was analyzed for lactose, protein, fat, and total solids with a fully automatic milk analyzer (Milko Scan™ FT+ Analyzer, Foss). The milk samples were analyzed for immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) using immunoglobulin-specific kits (Jinma Biological Engineering Co., Ltd., Shanghai, China).

RNA extraction, cDNA library construction and sequencing

Total RNA was isolated from the placenta using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The extracted RNA was treated with DNase I (Takara Biotechnology, China) for 45 min at 37 °C to remove residual DNA. The RNA concentration and integrity were measured using an Ultrasec™ 2100 pro UV/Visible Spectrophotometer (Amersham Biosciences, Uppsala, Sweden) and gel electrophoresis. Equal amounts of high-quality RNA from each specimen were pooled for RNA-Seq library construction. A cDNA library was prepared with a TruSeq RNA sample preparation kit following the manufacturer's instructions (Illumina) and sequenced on an Illumina HiSeq™ 2500 platform in 100 pair-ended mode (Biomarker Technologies).

De novo transcriptome assembly and functional annotation

To obtain clean reads, the raw reads were filtered by removing the adapter, poly-N and low-quality sequences. De novo assembly was performed using the Trinity method [18]. The K-mer and group pairs distance were set at 25 and 300, respectively, while the other parameters were set at default levels. Based on their overlap regions, the short reads were assembled into longer contigs, which were then clustered and further assembled into unigenes with the paired-end information. For gene functional annotation, all of the assembled transcripts were aligned to the publicly available protein databases, including the National Center for Biotechnology Information (NCBI), non-redundant protein (Nr), the Swiss-Prot protein, Gene Ontology (GO) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl), Clusters of Orthologous Groups (COG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/kegg2.html) using BLASTx analysis with a cut-off E-value of 10<sup>−5</sup>.

Differentially expressed genes (DEGs) analysis

Fragments per kilobase of transcript per million fragments mapped (FPKM) was calculated to represent the expression abundance of the unigenes. FPKM may reflect the molar concentration of a transcript by normalizing for RNA length and for the total read number. DEGs between PQQ treated and control samples were
identified by EBSeq. An FDR (false discovery rate) < 0.05 and |fold change (FC)| ≥ 2 was set as the threshold for significantly different expression.

\[
\text{FPKM} = \frac{\text{cDNA fragments}}{\text{Mapped fragments(millions)} \times \text{Transcript length(kb)}}
\]

**Quantitative real-time PCR (qRT-PCR)**

Total RNA from each sample was converted into cDNA using the Prime Script RT reagent Kit (TaKaRa Bio Catalog), and the cDNA was used for qRT-PCR. GAPDH was used as an internal control gene, and it did not respond to dietary treatments. The primer sequences are shown in Table 2. qRT-PCR was performed using the SYBR Green I Kit (TaKaRa Bio Catalog). For analyses, using an ABI PRISM 7500 SDS thermal cycler, PCRs were performed with 2.0 mL of first-strand cDNA and 0.4 mL of forward and reverse primers in a final volume of 20 mL. Samples were centrifuged briefly and run on the PCR machine using the default fast program (1 cycle at 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 34 s). All of the PCRs were performed in triplicate. The relative gene expression levels were determined using the \(2^{-\Delta\Delta Ct}\) method.

**Statistical analysis**

All data analyses were performed with SPSS 19.0 software (IBM-SPSS Inc., Chicago, Illinois, USA). The data were analyzed by One-way analysis of variance (ANOVA), and multiple comparisons were analyzed with the Tukey’s test in SPSS. The individual sow and her litter were defined as the experimental unit. The results were presented as the mean values and the standard error of the mean (SEM). In all statistical tests used, \(P < 0.05\) was considered significantly different.

**Results**

**Reproductive performance**

The results pertaining to the reproductive performance of sows are shown in Table 3. The back-fat thickness of sows at d 0 and 90 of gestation and at weaning did not differ between the two dietary treatments \((P > 0.05)\). Dietary PQQ·Na2 supplementation in the gestation and lactation diets had no effect on back-fat thickness gain during gestation or loss during lactation \((P > 0.05)\). The total piglets born, number of piglets born alive and born alive litter weight were significantly increased \((P < 0.05)\) by dietary PQQ·Na2 supplementation during gestation and lactation. The number of piglets weaned, litter weaning weight and piglet weaning weight were not affected by dietary PQQ·Na2 during gestation and lactation. \((P > 0.05)\).

**Antioxidant status and the concentration of nitric oxide**

The effects of dietary PQQ·Na2 supplementation during gestation and lactation on the antioxidant capacity in the placenta of sows are shown in Fig. 1. PQQ·Na2 dietary supplementation in gestation and lactation significantly increased the activities of SOD \((P = 0.011)\) and GSH-Px \((P = 0.032)\). The MDA \((P = 0.018)\) activity in the placenta after PQQ·Na2 supplementation was significantly lower than the control treatment. However, the CAT activity of the placenta was not significantly different.

### Table 2 Primers used for Real-time PCR

| Genes     | Primer sequence (5′→3′) | Product size, bp | GenBank No.  |
|-----------|-------------------------|-----------------|-------------|
| GAPDH     | F: ATGTTGAGGCTGGAGTGAA  | 155             | NM_001206359.1 |
|           | R: CCGTGCGTGGAAATCCACTG |                 |             |
| SOD1      | F: TCCATGTCCATCGTATTTGGA | 131            | NM_001190422.1 |
|           | R: AGTCACTTTGCCAGGTCTC |                 |             |
| IL-6      | F: AGCAAGGAGGACTGGCAGA  | 257             | NM_001252429.1 |
|           | R: GTGGTGGCTTTGTCTGGAAT |                 |             |
| IL-8      | F: ACTTCAAAGACTGCTGTTGCG | 120            | NM_213867.1  |
|           | R: RGGAATGCTTATTTAGCACTGG |                 |             |
| NOS2      | F: CGTATGCCACCAACAATGG  | 134             | NM_001143690.1 |
|           | R: GTGCCATACGGCAGCTGTA |                 |             |
| CDX2      | F: GTGCTTGATACCCATCCGG  | 110             | NM_001278769.1 |
|           | R: GATTCTCTCTCTCTCCCT |                 |             |
| CCN1      | F: TCAGCATGGCAGGAAAAAGGGCA | 122          | NM_001012022.1 |
|           | R: TGCCAGCGGAAGGCCACATT |                 |             |
| GCLC      | F: GCAATGGCCACCTCCTCGAG | 135            | XM_021098556.1 |
|           | R: GGAGGCTTAACTTCCGTCGTC |                 |             |
| CALM      | F: GCTGTCACCCAGGAGTCAGAAAG | 84           | XM_005668226.3 |
|           | R: GCTGTCACCCAGGAGTCAGAAAG |                 |             |

**IL-6 interleukin 6; IL-8 interleukin 8; SOD1 superoxide dismutase 1; NOS2 nitric oxide synthase 2; CDX2 caudal type homeobox 2; CCN1 cellular communication network factor 1; GCLC glutamate-cysteine ligase catalytic subunit; CALM calmodulin**
different \( (P > 0.05) \). The results of the antioxidant status in the plasma of sows are presented in Fig. 2. On d 90 of gestation, the activities of CAT \( (P = 0.029) \), SOD \( (P = 0.041) \) and GSH-Px \( (P = 0.030) \) were significantly increased by PQQ·Na\(_2\) supplementation. The MDA \( (P > 0.05) \) activity of the placenta was not significantly changed. On d 21 of lactation, the SOD \( (P = 0.027) \) and GSH-Px \( (P = 0.023) \) activities were significantly increased and the MDA \( (P = 0.023) \) activity was significantly decreased by PQQ·Na\(_2\) supplementation. The concentration of NO \( (P = 0.020) \) and iNOS \( (P = 0.037) \) were significantly increased in plasma on d 90 of gestation by PQQ·Na\(_2\) supplementation are shown in Fig. 3. PQQ·Na\(_2\) dietary supplementation in gestation and lactation significantly increased the concentration of NO \( (P = 0.034) \) and iNOS \( (P = 0.016) \) in the placenta of sows.

The colostrum

Figure 4 shows the protein, lactose, fat and total milk solids content of the sow milk. The concentrations of protein \( (P = 0.011) \) and total solids \( (P = 0.040) \) were significantly increased by PQQ·Na\(_2\) supplementation. The concentrations of fat and lactose were not significant \( (P > 0.05) \). The SOD \( (P = 0.011) \) and GSH-Px \( (P = 0.016) \) activity in milk from the PQQ sows was higher than that in milk from the Con sows, as shown in Fig. 5. The effects of dietary PQQ·Na\(_2\) supplementation during gestation and lactation on immunoglobulin concentrations in the colostrum of the sows are presented in Fig. 6. The concentrations of IgA, IgG and IgM were significantly \( (P < 0.05) \) increased in the colostrum with PQQ·Na\(_2\) supplementation.

| Item                                | Con   | SEM  | PQQ   | SEM  | \( P \)-value |
|-------------------------------------|-------|------|-------|------|--------------|
| Reproductive performance            |       |      |       |      |              |
| Total piglets born                  | 11.53 | 0.42 | 12.93 | 0.46 | 0.024        |
| Number of piglets born alive        | 10.67 | 0.32 | 11.87 | 0.37 | 0.022        |
| Born alive litter weight, kg        | 15.58 | 0.57 | 17.50 | 0.64 | 0.036        |
| Number of piglets weaned            | 9.60  | 0.31 | 10.2  | 0.41 | 0.260        |
| Litter weaning weight, kg           | 52.16 | 1.60 | 55.75 | 1.79 | 0.152        |
| Piglet weaning weight, kg           | 5.43  | 0.13 | 5.47  | 0.19 | 0.791        |
| Sow back-fat thickness, mm          |       |      |       |      |              |
| Gestation (d 0)                     | 14.29 | 0.44 | 14.22 | 0.31 | 0.903        |
| Gestation (d 90)                    | 15.77 | 0.30 | 16.13 | 0.38 | 0.475        |
| Gain                                | 1.49  | 0.23 | 1.91  | 0.32 | 0.393        |
| Parturition                         | 17.36 | 0.28 | 17.57 | 0.34 | 0.602        |
| Weaning                             | 15.81 | 0.24 | 15.80 | 0.19 | 0.956        |
| Loss                                | 1.55  | 0.20 | 1.77  | 0.30 | 0.547        |

ab Within a row, means without a common superscript differ \( (P < 0.05) \) \( n = 15 \).

SEM, Standard error of the mean
High-throughput transcriptome sequencing and de novo assembly
To understand the molecular basis of the difference in the reproductive performance between Con and PQQ, the placenta was used to build 6 libraries for high-throughput sequencing. We obtained a total of 54.18 Gb of raw data for the 6 samples (Table 4). We discarded low-quality reads, which contained adapters and unknown or low-quality bases, and after stringent quality checks and data cleaning, the clean reads were obtained (Table 4). The GC (guanine + cytosine) contents of these samples were 51.86–53.06%, with an average of 52.51%.

The average Q20 and Q3 percentages reached 97.85% and 94.51%, respectively (Table 4).

Gene annotation and functional classification
All unigenes were aligned to 7 protein databases, including COG, GO, KEGG, KOG, Swiss-Prot, and Nr, using BLASTx with an E-value threshold of $10^{-5}$ and Pfam using HMMER with an E-value threshold of $10^{-10}$. As shown in Table 5, of 462 unigenes annotated, 448 (96.97%) unigenes had significant BLASTx matches in the Nr database. Based on comparison against the Swiss-Prot database, 370 (80.09%) unigenes had significant matches.
Fig. 4 Effects of dietary PQQ·Na₂ supplementation during gestation and lactation on the colostrum analysis in milk. a protein; b fat; c total solids; d lactose. All values are expressed as means ± SEM (n = 6). a, b Mean values within a column with unlike superscript letters were significantly different (P < 0.05).

Fig. 5 Effects of dietary PQQ·Na₂ supplementation during gestation and lactation on antioxidant status in milk of sows. Con, control treatment; PQQ, PQQ·Na₂ treatment; a CAT, catalase; b SOD, superoxide dismutase; c MDA, malondialdehyde; d GSH-Px, glutathione peroxidase. All values are expressed as means ± SEM (n = 8). a, b Mean values within a column with unlike superscript letters were significantly different (P < 0.05).
In the Pfam and GO databases, 415 (89.93%) and 384 (83.12%) unigenes were also found to have significant matches, respectively, and 316 (68.40%) unigenes were similar to proteins in the KEGG database. To further evaluate the completeness of our transcriptome library and the effectiveness of our annotation process, we searched the annotated sequences for genes with COG (cluster of orthologous groups) classifications, and 145 unigenes were assigned to the COG classification (Fig. 7). Among the 25 COG categories, the cluster for “General function prediction only” (24, 15.19%) represented the largest group, followed by “Carbohydrate transport and metabolism” (20, 12.66%) and “Posttranslational modification, protein turnover, chaperones” (19, 12.03%). Gene ontology (GO) was also used to classify the functions of the predicted unigenes. Based on the sequence homology, 384 sequences were categorized into 61 functional groups (Fig. 6). The assigned functions of the unigenes covered a broad range of GO categories. The unigenes were assigned to three main categories, including the cellular component, molecular function, and biological process categories (Fig. 8). Reproduction (27, 7.03%) and reproduction process (27, 7.03%) were assigned to the biological process category.

Analysis of the differentially expressed unigenes (DEGs) by RNA-seq

The gene expression levels of DEGs in the placenta were measured using the calculated values of the RPKM parameter (reads per kilobase of exon per million reads mapped). The TMM (trimmed mean of M-values) method was used to standardize the read counts. Thereafter, the differentially expressed unigene analysis was performed using EB Seq. An absolute value of log₂ fold change (FC) ≥2 and a false discovery rate (FDR) value of < 0.05 were employed to identify the DEGs and to explore the gene expression levels of DEGs in the placenta between the Con treatment and PQQ treatment. As shown in Additional file 1: Table S1, 462 unigenes were differentially expressed between the Con treatment and PQQ treatment. Among these unigenes, 199 were upregulated, while 263 unigenes were downregulated. By matching DEGs to the KEGG database, we identified 29 pathways (P < 0.05, Table 6).

Validation of differentially expressed genes by qRT-PCR

To validate the results of RNA-seq analysis, qRT-PCR was employed to determine the relative expression of 8 genes in the placenta (Fig. 9), including SOD1, IL6, IL8, NOS2, CDX2, CCN1, GCLC and CALM. IL6, IL8 and CCN1 were significantly downregulated. SOD1, NOS2, CDX2, GCLC and CALM were upregulated by PQQ-Na₂ dietary supplementation (P < 0.05), which was consistent with the data from the RNA-seq analysis. Differences in the magnitude of fold-change values were likely due to differences in detection sensitivity of the two methods. In addition, correlation analysis demonstrated that the values of log₂ (fold-change) obtained from RNA-seq and qRT-PCR were significantly correlated (R² = 0.94). Thus, our RNA-seq analysis results are valid.

Table 4 Summary of sequencing and assembly data

| Item | Sample ID | Clean base | Clean read | GC, % | Q20, % | Q30, % |
|------|-----------|------------|------------|-------|-------|-------|
| Con  | Con1      | 8,831,393,714 | 29,527,850 | 51.86 | 97.27 | 93.57 |
|      | Con2      | 6,702,986,510 | 22,509,861 | 53.02 | 97.91 | 94.66 |
|      | Con3      | 10,898,101,426 | 36,518,053 | 52.23 | 97.98 | 94.64 |
| PQQ  | PQQ1      | 9,902,483,500 | 33,262,116 | 53.06 | 98.10 | 94.92 |
|      | PQQ2      | 7,221,391,084 | 24,187,833 | 52.37 | 97.88 | 94.59 |
|      | PQQ3      | 7,373,706,268 | 24,771,902 | 52.52 | 97.97 | 94.73 |
Discussion

The use of PQQ in nutrition is increasingly being discussed in the literature [19, 20]. Jonscher KR et al. [21] reported that PQQ treatment led to a significant increase in placental weight and placental surface area. Yin et al. [22] suggested that the dietary supplementation of 1.50 mg/kg PQQ·Na2 is the lowest functional dose to improve the growth performance for weaned pigs and the expression of the jejunal tight junction protein ZO-1 was significantly higher in pigs with PQQ·Na2 supplementation. Zhang et al. [23] suggested that dietary 1.6 mg/kg PQQ·Na2 supplementation during gestation and lactation of female rats can significantly increase the number of viable foetuses per litter, born alive litter weight and the mRNA expression levels of CAT, GPX2 and SOD1 in the placenta. Steinberg et al. [24] observed that 0.4 mg PQQ/kg in an amino acid-based diet optimized reproduction. To our knowledge, this is the first study to examine the effects of dietary PQQ·Na2 supplementation during gestation and lactation in sows. Several reports have investigated DEGs from reproductive tissues, such as the endometrium, in pigs [25]. DEGs in the placenta have rarely been demonstrated using RNA-seq.

During pregnancy and lactation in sows, high energy and oxygen levels are required to satisfy increasing metabolic burdens for fetal growth, placenta development and milk production, which could cause elevated ROS production [26]. Oxidative stress results from increased production of reactive oxygen species (ROS) or a decrease in antioxidant defense. Oxidative damage is a strong indicator of the health status and wellbeing of animals [27]. A recent study showed that pregnant sows had elevated oxidative stress during late gestation and lactation, which was responsible for impaired milk production, reproductive performance, and longevity of sows [28]. The protective role of an antioxidant against oxidative stress in sows has been clearly demonstrated [29], and dietary addition of antioxidants can reduce oxidative stress and improve the reproductive performance of sows [25]. Thus, dietary antioxidant concentrations need to be added in sow diets, especially to prevent excessive oxidative stress during gestation and lactation. In this study, we found that the antioxidant status of sows, including in the plasma, placenta and milk, was partially improved and oxidative stress markers were partially reduced by dietary PQQ·Na2 supplementation. SOD is known to serve a protective function for the elimination of reactive free radicals and, therefore, it represents an important antioxidant defense in nearly all cells exposed to oxygen. GSH forms an important part of the nonenzymatic antioxidants [30]. Similar to other sulfhydryl-containing products, GSH also has regulatory and protective roles in the body, as it establishes the defenses of the body against tissue injury due to chemicals through...
its ROS scavenging, cell viability and membrane-stabilizing effects [31]. In this study, dietary PQQ·Na₂ supplementation can increase the antioxidant enzyme activities of SOD and GSH-Px in the placenta, plasma and milk. A previous study has demonstrated that SOD activity can be upregulated by PQQ·Na₂ treatment [32], and this is consistent with our study results. The antioxidant enzymes SOD and CAT are considered the first line of defense against ROS. In this study, CAT was increased in the plasma (90 d of gestation). MDA is an end product of free-radical chain reactions and lipid peroxidation [33], so it is frequently used in the measurement of lipid peroxide levels, and it correlates well with the degree of lipid peroxidation [34]. In the present study, the MDA levels in the placenta and plasma (21 d of lactation) was significantly decreased by PQQ·Na₂ supplementation. PQQ acts as an antioxidant by scavenging O₂⁻ and protects mitochondria from oxidative stress-induced damage. In our study, the total piglets born, number of piglets born alive and born alive litter weight were significantly increased. Evidence has shown that supplementation with antioxidants, such as selenium, vitamin E and vitamin C, improves antioxidant status and reproductive performance in sows [35, 36]. Similarly, a growing number of studies have demonstrated that certain functional substances, such as chitosan [37], resveratrol [38], and isoflavone [25], alleviate oxidative stress and improve the reproductive performance of sows. Our results are in alignment with these studies and demonstrate that dietary PQQ·Na₂ exerts a beneficial role in antioxidant defense and the reproductive performance of sows.

Colostrum is of great importance for the growth and development of piglets during and after lactation [39]. Because neither creep feed nor milk replacer was used for the suckling piglets in this study, the sow milk served as the sole source of nutrients and antioxidants for the piglets. Therefore, litter performance reflected the nutrient composition and antioxidant status of the colostrum and sow milk only. There is evidence that sow's colostrum and milk include various antioxidants, including SOD, GSH-Px and GSH [25, 40]. In the present study, SOD and GSH-Px activity in the colostrum was significantly increased by dietary PQQ·Na₂ during gestation and lactation. Milk antioxidants, which provide antioxidant protection to suckling piglets, may be as important as nutrients or immunological factors in protecting the health of the neonatal piglet [35]. Colostrum is the main external resource providing piglets with nutrients and...
maternal immune molecules. Newborn piglets can hardly obtain passive immunity from the maternal blood during the fetal period because of the special epitheliochorial structure of the pig placenta. Before their own immune system is fully developed, colostrum is the sole external resource which provides piglets with nutrients and maternal immune molecules [41]. In the present study, the concentrations of protein and total solids were increased. The high total solids and protein content in colostrum is mostly due to immunoglobulin [42]. Immunoglobulin in colostrum, mainly IgG, provides humoral immune protection for the newborn piglet until its own immune system has sufficiently matured to respond to antigens [43]. Additionally, immunoglobulin in the colostrum and milk could increase susceptibility to infection in newborn animals, not only in the immediate postnatal period but also after weaning [44]. IgA is the main immunoglobulin of milk and could protect piglets against local pathogens, commensal bacteria and food antigens in the digestive tract [45]. In our study, the concentrations of IgG, IgA and IgM in the colostrum were significantly increased by PQQ·Na2 supplementation, which might aid the humoral and mucosal immunity of piglets.

Nitric oxide (NO) is a pleiotropic regulator and is pivotal to numerous biological processes, including vasodilation, neurotransmission, and macrophage-mediated immunity [46]. NO plays an important role in regulating placental-fetal blood flow, contributing to maternal systemic vasodilation during pregnancy and regulating uterine and fetal placental blood flow [47]. It has been reported that antioxidant activity inhibits lipid peroxidation, increases nitric oxide (NO) production, reduces oxidation of low-density lipoproteins, and preserves

| Table 6 KEGG pathway enrichment of DEGs |
|----------------------------------------|
| KEGG_pathway                          | KO_ID       | P-value     |
| Steroid biosynthesis                  | ko00100     | 3.39E-06    |
| Transcriptional misregulation in cancer | ko05202   | 4.12E-05    |
| Lysosome                              | ko04142     | 0.000590824 |
| TGF-beta signaling pathway            | ko04350     | 0.000855626 |
| Bladder cancer                        | ko05219     | 0.001822117 |
| Malaria                               | ko05144     | 0.00268114  |
| Jak-STAT signaling pathway            | ko04630     | 0.003155387 |
| HIF-1 signaling pathway               | ko04066     | 0.003863436 |
| Signaling pathways regulating pluripotency of stem cells | ko04550 | 0.005510021 |
| Other glycan degradation              | ko00511     | 0.006945803 |
| Pertussis                             | ko05133     | 0.010337763 |
| Insulin signaling pathway             | ko04910     | 0.01140163  |
| FoxO signaling pathway                | ko04068     | 0.013399779 |
| Cytokine-cytokine receptor interaction | ko04060   | 0.015031804 |
| TNF signaling pathway                 | ko04668     | 0.015490522 |
| Terpenoid backbone biosynthesis       | ko00000     | 0.015624507 |
| Renin secretion                       | ko04924     | 0.019634552 |
| Salmonella infection                  | ko05132     | 0.0219693  |
| Mucin type O-Glycan biosynthesis      | ko00512     | 0.028443139 |
| Hippo signaling pathway               | ko04390     | 0.029186826 |
| Complement and coagulation cascades   | ko04610     | 0.031260074 |
| Amino sugar and nucleotide sugar metabolism | ko00520 | 0.031819731 |
| African trypanosomiasis              | ko05143     | 0.035721873 |
| ABC transporters                      | ko02010     | 0.042072163 |
| Regulation of lipolysis in adipocytes | ko04923    | 0.04321741  |
| AGE-RAGE signaling pathway in diabetic complications | ko04933 | 0.046396257 |
| Insulin resistance                    | ko04931     | 0.048015681 |
| Folate biosynthesis                   | ko00790     | 0.048299174 |
| Chagas disease (American trypanosomiasis) | ko05142 | 0.049669742 |
superoxide dismutase (SOD) activity [48]. In the present study, PQQ increased antioxidants and iNOS in the placenta and plasma, which can increase NO. The increased NO plays an important part in improving placental vascular function and promoting the nutrient supply to the fetus. NO plays an important role in vasodilatation and regulates uterine blood flow, promoting the transfer of nutrients to the fetus [49]. It has reported that NO is a key regulator of angiogenesis and embryo development [50]. In previous studies we showed that PQQ regulates intracellular JAK/STAT signaling pathway activation. Eskouhie Tchaparian et al. reported changes in gene expression patterns and transcriptional networks that respond to dietary PQQ restriction or pharmacological administration, they found JAK/STAT pathways seem particularly influenced by PQQ [57]. Zhang reported that the insulin signaling pathway was down-regulated in the placenta of women with gestational diabetes mellitus [58]. Dysregulation of Hippo signaling component genes can result in embryonic lethality [59]. Folates are needed for fetal growth and placental development, since they activate cell growth and biosynthetic processes that are essential during pregnancy [60].

GO analyses showed that 27 DEGs were involved in reproduction. Among these DEGs, the ovo-like zinc finger 2 (OVOL2), distal-less homeobox 1 (DLX1), distal-less homeobox 2 (DLX2), distal-less homeobox 5 (DLX5), msh homeobox 2 (MSX2), caudal type homeobox 2 (CDX2), nitric oxide synthase 2 (NOS2) and calmodulin (CALM) were upregulated. Unezaki reported that ovol2 function is required for endothelial cell growth during heart development and angiogenesis of extraembryonic and embryonic vessels [61]. The Dlx genes encode a family of transcription factors with important roles in patterning and differentiation during vertebrate embryogenesis [62]. Previous studies indicate that MSX2 plays an important role in mammalian embryonic diapause [63, 64]. Sakurai suggested that CDX2 is essential for early development and gene expression and is involved in differentiation of the inner cell mass and trophectoderm lineages in embryos [65]. Kwon showed, by comparing sperm proteins from

**Figure 9** Validation of RNA-seq results with qRT-PCR analysis. The mRNA levels of selected genes were analyzed by qRT-PCR and normalized to GAPDH. IL-6, interleukin 6; IL-8, interleukin 8; SOD1, superoxide dismutase 1; NOS2, nitric oxide synthase 2; CDX2, caudal type homeobox 2; CCN1, cellular communication network factor 1; GCLC, glutamate-cysteine ligase catalytic subunit; CALM, calmodulin. All values are expressed as means ± SEM (n = 8). a, b, c Mean values within a column with unlike superscript letters were significantly different (P < 0.05).
different litter sizes, that CALM was highly expressed in high swine litter sizes and was positively related to litter size [66]. Furthermore, antioxidant genes, such as SOD1, GCLC and DHCR24, were also upregulated by PQQ·Na2 supplementation. In sows, supplementation improves antioxidant levels and alleviates oxidative stress effectively, which are beneficial to litter size and piglet growth. DHCR24 exerts cytoprotective effects against endoplasmic reticulum stress by eliminating ROS. DHCR24 can scavenge hydrogen peroxide (H2O2), protecting cells from oxidative stress-induced apoptosis [67]. GCLC is an important part of GSH, which is an extremely important antioxidant. It not only scavenges free radicals but also maintains the redox-sensitive active sites of many enzymes from an oxidized form to a reduced form [68]. The supply of glucose to the embryo from the maternal circulation is important for normal metabolism and growth, as glucose constitutes the main energy substrate during embryogenesis [69]. The solute carrier (SLC) family (including SLC1A1, SLC7A4, SLC7A10 and SLC19A1) was upregulated, and many nutrient carriers and growth factors decrease as the dam is exposed to stress. Oxidative stress, defined as an imbalance between the production of free radicals and reactive metabolites, is closely related to inflammation. GO analysis identified genes involved in the inflammatory response (including IL6, IL8, IL11 and CCN1) that were downregulated. KEGG pathway analysis showed that the cytokine-cytokine receptor interaction pathway was significantly reduced, which indicated that the inflammatory state of the placenta was alleviated by maternal PQQ·Na2 supplementation. A previous study showed that PQQ can reduce the expression of inflammatory cytokine genes [20] which was consistent with our study.

Conclusions
In conclusion, our results have shown that dietary 20 mg/kg PQQ·Na2 supplementation during gestation and lactation in sows can significantly increase the total piglets born, number of piglets born alive and born alive litter weight. It also increased antioxidant levels in the placenta, plasma and milk. The concentration of NO was significantly increased in the plasma and placenta. RNA-seq analysis showed that 462 unigenes were differentially expressed between Con treatment and PQQ treatment. Among these unigenes, 199 were upregulated, while 263 unigenes were downregulated. By matching DEGs to the KEGG database, we identified 29 pathways. These provide a theoretical basis to further explore the effect of PQQ on the reproductive performance mechanism of sows. The present study can provide a scientific basis for dietary PQQ·Na2 supplementation in sows.

Additional file

**Additional file 1**: Table S1. Analysis of the differentially expressed unigenes (DEGs) by RNA-seq. (DOC 644 kb)

**Abbreviations**
ANOVA: Analysis of variance; CALM: Calmodulin; CAT: Catalase; CCN1: Cellular communication network factor 1; CDX2: Caudal type homeobox 2; CDX2: Caudal type homeobox 2; COG: Clusters of Orthologous Groups; Con: Contra; DEGs: Differentially expressed genes; DHCR24: 24-dehydrocholesterol reductase; DLX1: Distal-less homeobox 1; DLX2: Distal-less homeobox 2; DLX5: Distal-less homeobox 5; FC: Fold change; FDR: False discovery rate; FPKM: Fragments per kilobase of transcript per million fragments mapped; GCLC: Glutamate-cysteine ligase catalytic subunit; GO: Gene Ontology; GSH-Px: Glutathione peroxidase; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IL-11: Interleukin 11; IL-6: Interleukin 6; IL-8: Interleukin 8; INOS: Inducible NOS; KEGG: Kyoto Encyclopedia of Genes and Genomes; MDA: Malondialdehyde; MSX2: msh homeobox 2; NCBI: National Center for Biotechnology Information; NO: Nitric oxide; NOS2: Nitric oxide synthase 2; Nr: Non-redundant protein; OVOL2: Ovo like zinc finger 2; PQQ: Pyrroloquinoline quinone; PQQ·Na2: Pyrroloquinoline quinone disodium; qRT-PCR: Quantitative real-time PCR; RNA-seq: RNA-seq; ROS: Reactive oxygen species; SEM: Standard error of the mean; SLC19A1: Solute carrier family 19 member 1; SLC1A1: Solute carrier family 1 member 1; SLC7A10: Solute carrier family 7 member 10; SLC7A4: Solute carrier family 7 member 4; SOD: Superoxide dismutase; SOD1: Superoxidismutase 1

**Acknowledgements**
The authors would like to thank the Mudanjiang Dawan Animal Husbandry Company with limited liability for animal management.

**Authors’ contributions**
All authors participated in the development of the study concept and design; B. Z., C. W. and W. Y. were responsible for the execution of the study; S. B., S. H. and Q. M. were involved in the animal experiments, analysis and data collection; H. Z. and C. W. were responsible for the statistical analysis; B. Z. wrote the draft of the manuscript; Q. M., A. S. and S. B. reviewed and revised the manuscript. None of the authors has any conflicts of interest to declare. All authors read and approved the final manuscript.

**Funding**
This work was supported by the National Key Research and Development Plan of China (2016YFD0501207), the China Agriculture Research System (CARS-36).

**Availability of data and materials**
The datasets produced and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**
The protocols used in this experiment were approved by the Northeast Agricultural University Institutional Animal Care and Use Committee. All animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Received: 11 February 2019 Accepted: 5 June 2019
Published online: 07 August 2019

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