Peroxiredoxin 4 as potential fertility marker in boars

Do-Yeal Ryu
Chung-Ang University
Won-Ki Pang
Chung-Ang University
Md Saidur Rahman
Chung-Ang University
Yoo-Jin Park
Chung-Ang University
Myung-Geol Pang (mgpang@cau.ac.kr)
Chung-Ang University https://orcid.org/0000-0003-2019-3789

Research

Keywords: peroxiredoxin, spermatozoa, male fertility, biomarker

Posted Date: March 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-19365/v1

Abstract

Background: High levels of reactive oxygen species are toxic to spermatozoa as they induce DNA damage, which leads to male infertility. Therefore, reduction of oxidative stress using reducing agents, such as antioxidant enzymes, is required. Peroxiredoxins (Prdxs) are known to play a critical role in the regulation of male fertility as antioxidant enzymes. Although several studies have suggested a close association between Prdxs and male fertility, few studies have explored the efficacy of Prdxs to predict fertility. Therefore, the current study was designed to discover the most sensitive biomarkers among the Prdxs with six isoforms, and furthermore to determine whether Prdxs are more suitable fertility markers for boar spermatozoa compared to conventional semen analysis.

Results: The mRNA levels of *PRDXs* and several sperm parameters were examined in spermatozoa collected from 20 boars with different litter sizes. Our study showed that there was a significant positive correlation between the litter size and the levels of *PRDX 4* among all isoforms in spermatozoa. Subsequently, a regression analysis using a combination of markers was conducted to increase efficacy for fertility prediction. According to the data, *PRDX4* had the highest efficacy compared to other combination models. The prediction accuracy of male fertility was further evaluated through receiver operating characteristic curve analysis, which showed that *PRDX 4* could predict the litter size with a high overall accuracy of 95%.

Conclusions: As fertility biomarkers, genomic markers might be more accurate for predicting male fertility compared to conventional semen analysis. Our findings indicate that *PRDX 4* might be a potential biomarker for diagnosing male fertility and subsequently improving reproductive efficacy in boars.

Background

It is widely known that high levels of reactive oxygen species (ROS) are toxic to spermatozoa owing to DNA damage, which leads to apoptotic-like changes in these cells(1-5). This affects sperm motility, morphology, and function, all of which affect male fertility. Compared to other cell types, spermatozoa are particularly sensitive to ROS due to their restricted antioxidation ability(1-4, 6, 7). For this reason, substantial research has been focused on antioxidants to suppress oxidative stress in recent years. Dandekar et al. reported that antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, protect spermatozoa from ROS-induced pathologies(8). Another recent study demonstrated that catalase exerts a protective role to restore sperm quality(9, 10). In agreement with these findings, some studies have reported the differential expression of antioxidant enzymes between fertile and infertile men(11-13). Consistent with these evidences, our previous proteomic study demonstrated that the sperm proteome changes during capacitation, which is one of the fundamental processes that spermatozoa undergo to acquire the ability to fertilize an egg(14). Furthermore, this analysis revealed that peroxiredoxin (Prdx) 5 is highly expressed in capacitated spermatozoa. Previously, Prdxs have been reported to be vital antioxidant enzymes in spermatozoa and one of the most common antioxidant enzymes that counter reactive oxygen species (ROS) in almost all cell types(2, 14-17). Similarly, another proteomic study demonstrated that the levels of Prdxs are reduced in spermatozoa of infertile male patients (13). In addition, an extensive literature research revealed that the abnormal function of Prdxs affects sperm motility, capacitation, mitochondrial membrane potential, and DNA integrity(2, 14-17). Thus, the levels or activities of Prdxs in spermatozoa may serve as a potential target for diagnosing male fertility. While a large body of knowledge supports the close association between Prdxs and male fertility, the likelihood of Prdxs to predict fertility is still lacking, particularly in a clinical setting. In addition, investigation of particular isotypes of *PRDX* that could specifically define fertility levels is also of great importance.

Many limitations are encountered when conducting research on humans to diagnose and predict diseases, such as infertility, due to various reasons (i.e., a variety of etiologies, unexplained etiologies, limited number of reproductive trials, sample size, ethical issues, etc.). Therefore, many studies have been conducted on swine samples due to their advantages as a biomedical model. Various genetic traits related to fertility have been well established in swine, which is thus a very effective biomedical model(18-21). Moreover, the fertilizing ability of a human male can only be evaluated with one woman, whereas pigs can be used as a better fertility model as artificial insemination (AI) allows one to obtain a variety of fertility outcomes by breeding with many females with different genetic traits.

Therefore, the goals of our study were i) to better elucidate the role of each *PRDX* in male fertility, for which, the expression of *PRDXs* was measured and compared with clinical fertility data for pig, ii) to elucidate the efficacy of *PRDXs* as biomarkers for predicting the male fertility, and iii) to develop the most useful biomarker models using a combination of *PRDX* isoforms and conventional methods, as alternatives to independent approaches for predicting male fertility.

Methods

Animal and semen collection

Yorkshire boar (range = 13 to 25 months) semen was collected using the gloved-hand technique twice per week. Collected semen was transferred onto ice from the Grand-Grandparents Farm (Sunjin Co., Danyang, Korea) to the laboratory within 2 h(22). This experiment was performed in two stages. i) Semen samples were collected from 10 boars at the Grand-Grandparents Farm (Sunjin Co., Danyang, Korea). Depending on boar litter sizes, samples were categorized into high (n = 5, average = 13.62 ± 0.25) and low-litter size groups (n = 5, average = 11.34 ± 0.32). Each semen sample was centrifuged at 500 ×*g* for 20 min using a discontinuous Percoll gradient (70% [v/v] and 35% [v/v] with mTCM 199 medium) (Sigma-Aldrich, St. Louis, MO, USA) to discard seminal plasma and dead spermatozoa. After the Percoll gradient wash, live spermatozoa were cultured in modified tissue culture

medium 199 (mTCM 199; containing 0.91 mM sodium pyruvate, 3.05 mM d-glucose, 2.92 mM calcium lactate, and 2.2 g/L sodium bicarbonate; Sigma-Aldrich) and incubated for 30 min at 37 °C under 5% atmospheric CO_2(14, 23). To evaluate sperm functional parameters between the two groups, sperm motility, motion kinematics, sperm capacitation status, and mRNA and protein levels of Prdx4 were evaluated.) Unlike the first trial, we randomly collected 20 individual boar semen samples of unknown fertility. Correlation analysis and quality assessment were performed as described for the previous stage.

Computer-assisted sperm analysis (CASA)

Sperm motility (%) and motion kinematic parameters were examined using the CASA system (SAIS Plus version 10.1; Medical supply, Seoul, Korea)(24). Briefly, 10 µL of the sperm suspension was placed in a Makler chamber (Makler, Haifa, Israel) on a heated plate (at 37 °C). The SAIS software and 10× phase contrast objective microscope were used to analyze and detect spermatozoa. The CASA parameters were set as reported previously (frames acquired, 20; frame rate, 30 Hz; minimum contrast, 7; minimum size, 5; low/high size gates, 0.4–1.5; low/high intensity gates, 0.4–1.5; non-motile head size, 16; and non-motile brightness, 14). Hyperactivation (HYP) were identified as having curvilinear velocity (VCL) ≥ 150 µm/s, mean amplitude of head lateral displacement (ALH) ≥ 5 µm/s, and linearity (LIN) ≤ 50%(2, 25, 26).

Combined H33258/chlortetracycline fluorescence (H33258/CTC) assessment

H33258/CTC assessment was conducted to evaluate the capacitation/acrosome status of spermatozoa(2, 23). Briefly, 135 µL of each sample was incubated with 15 µL of H33258 solution for 10 min at room temperature. Then, 250 µL of 2% polyvinylpyrrolidone in Dulbecco's phosphate-buffered saline (DPBS) was added and centrifuged at 100 ×*g* for 2.5 min to remove the surplus dye. After that, the cell pellet was resuspended in 100 µL of DPBS and 100 µL of CTC solution. A microphot-FXA microscope was used to observe the capacitation status of spermatozoa using ultraviolet BP 340–380/LP 425 and BP 450–490/LP 515 excitation/emission filters for H33258 and CTC, respectively. The capacitation status was classified into non-capacitated (F pattern), capacitated (B pattern), and acrosome-reacted (AR pattern), as reported previously(2, 27). At least 400 spermatozoa were counted per slide.

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and TRIzol (Invitrogen) were used for RNA extraction from each spermatozoa group. Briefly, the prepared sperm pellets were lysed using the lysis buffer with 2% 2-mercaptoethanol (Sigma-Aldrich). Next, 200 µL of chloroform (Sigma-Aldrich) was added to samples and incubated at 20 °C for 5 min. Samples were then centrifuged at 12,000 ×g for 25 min. After centrifugation, 500 µL of the upper layer was moved into a new RNase-free tube along with an equal volume of 100% ethanol. The mixture was then transferred into a spin cartridge (Invitrogen) and centrifuged at 12,000 ×g for 15 s. Finally, to isolate the RNA, 20 µL of nuclease-free water was added to the spin cartridge. RNA concentration and 260/280 ratio were evaluated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Finally, PrimerSelect™ software (DNASTAR, Madison, WI, USA) was used to design primers for PRDX 1–6 and GAPDH (Supplementary table. 1). All primers were designed according to the Reference genome Sscrofa11.1 Primary Assembly. qRT-PCR was performed, and the results were analyzed using ABI PRISM 7500 (Applied Biosystems) and the $2^{-\Delta\Delta Ct}$ method(28).

Western blot analysis of total Prdx 4 and thiol-oxidized Prdx 4

Western blot analysis of total Prdx 4 and thiol-oxidized Prdx 4 in bovine spermatozoa was performed. Briefly, each sample was washed three times with DPBS and centrifuged at 10,000 × g for 10 min. The supernatant was removed, and sperm pellets were resuspended in Laemmli sample buffer (63 mm Tris, 10% glycerol, 10% sodium dodecyl sulphate, 5% bromophenol blue) with (reducing condition) or without (non-reducing condition) 100 mM DTT and incubated at room temperature for 10 min. After incubation, samples were centrifuged at 10,000 × g for 10 min, and cell pellets were boiled at 100°C for 3 min. Samples were resolved by SDS-PAGE using a 12% mini-gel system (Amersham, Piscataway, NJ, USA), and separated proteins were transferred to a polyvinylidene fluoride membrane (Amersham). The membrane was blocked for 1 h at room temperature with blocking agent (3%; Amersham). To detect Prdx 4 under both reducing and non-reducing conditions, the membrane was incubated overnight with a rabbit polyclonal anti-peroxiredoxin 4 antibody (1: 2,000; Abcam) overnight at 4°C(16). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abcam) diluted 1:5000 for 1 h at room temperature. α-tubulin was used as an internal control (detected with a mouse monoclonal anti-α-tubulin antibody, 1:10000; Abcam) for 2 h at room temperature. Membranes were washed three times with PBS-T, and protein-antibody complexes were visualized using enhanced chemiluminescence. Bands were scanned using a GS-800-calibrated imaging densitometer (Bio-Rad, Hercules, CA, USA) and analyzed using Quantity One software (Bio-Rad). The ratio of Prdx 4/α-tubulin was calculated.

Artificial insemination (AI)

We randomly selected 20 Yorkshire boars with unknown fertility from the Grand-Grandparents Farm to evaluate the correlation between litter size and sperm parameters or PRDX expression levels in individual samples. Selected semen was diluted to 30×10^6 sperm cells/100 mL with Beltsville Thawing Solution for AI(22, 23). Diluted boar semen samples were inseminated by an experienced artificial inseminator into sows (n = 568).

Quality assessment of markers

To determine the accuracy of predicting boar fertility based on the litter size, we evaluated the following five major parameters during the screening test: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy, which were determined according to the cut-off value associated with receiver operating curve (ROC) analysis as described previously (23, 27, 29, 30). Briefly, cut-off value was decided by the predicted probability that the individual semen appertained to [litter size ≥ 12] according to the marker(s) in logistic regression. Sensitivity, which is the true positive results, is calculated as the number of boars with predicted probability ≥0.5 to the number of boars with litter size ≥12 piglets. Specificity, which is the true negative results, is calculated as the proportion of the number of boars with predicted probability <0.5 to the number of boars with a litter size <12 piglets. The positive predictive value was decided as the proportion of the actual number of boars that had a litter size ≥12 piglets to the number of boars with a predicted probability ≥0.5. The negative predictive value was decided as the proportion of the actual number of boars that had a litter size <12 piglets to the number of boars with a predicted probability <0.5.

Marker combination and screening test

To evaluate multiple gene combinations, backward multiple regression analysis was performed. Backward multiple regression analysis conducted with a full model (all isoforms of *PRDXs,* HYP, and protein levels of Prdx 4) and at every single stage gradually removes variables from regression model to discover a reduced model that principal explains the data. We then calculated the combined biomarker score, depending on the following formulae:

Y1) HYP, mRNA levels of *PRDX 1 to 6*, and protein levels of Prdx 4 [Y = 8.641 - 0.003X1 (HYP score) + 0.074X2 (*PRDX 1* score) + 0.845X3 (*PRDX 2* score) - 1.256X4 (*PRDX 3* score) + 5.371X5 (*PRDX 4* score) + 0.194X6 (*PRDX 5* score) + 0.465X7 (*PRDX 6* score) - 0.451X8 (Prdx 4 protein score)].

Y2) HYP, mRNA levels of *PRDX 2 to 6*, and protein levels of Prdx 4 [Y = 8.661 - 0.003X1 (HYP score) + 0.839X2 (*PRDX 2* score) - 1.240X3 (*PRDX 3* score) + 5.363X4 (*PRDX 4* score) + 0.208X5 (*PRDX 5* score) + 0.477X6 (*PRDX 6* score) - 0.44X7 (Prdx 4 protein score)].

Y3) mRNA levels of *PRDX 2 to 6* and protein levels of Prdx 4 [Y = 8.681 + 0.843X1 (*PRDX 2* score) - 1.249X2 (*PRDX 3* score) + 5.298X3 (*PRDX 4* score) + 0.208X4 (*PRDX 5* score) + 0.473X5 (*PRDX 6* score) - 0.44X6 (Prdx 4 protein score)]

Y4) mRNA levels of *PRDX 2 to 4, 6*, and protein levels of Prdx 4 [Y = 9.107 + 1.067X1 (*PRDX 2* score) - 1.312X2 (*PRDX 3* score) + 5.298X3 (*PRDX 4* score) + 0.208X4 (*PRDX 5* score) + 0.473X5 (*PRDX 6* score) - 0.44X6 (Prdx 4 protein score)]

Y5) mRNA levels of *PRDX 2, 4, 6*, and protein levels of Prdx 4 [Y = 8.714 + 0.045X1 (*PRDX 2* score) + 5.127X2 (*PRDX 4* score) + 0.373X3 (*PRDX 6* score) - 0.44X4 (Prdx 4 protein score)]

Y6) mRNA levels of *PRDX 4, 6*, and protein levels of Prdx 4 [Y = 8.686 + 5.167X1 (*PRDX 4* score) + 0.409X2 (*PRDX 6* score) - 0.439X3 (Prdx 4 protein score)]

Statistical analysis

The data were analyzed using SPSS (v. 25.0; Chicago, IL). Pearson correlation coefficients were evaluated to determine the correlation between motility, motion kinematics, capacitation status, litter size, and *PRDX* expression levels. Backward multiple linear regression was used to identify the optimal combination conditions. The combined scores were then calculated according to the regression formulae described above. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the ability of individual parameters for predicting the litter size. cut-off value was decided by the predicted probability that the individual semen appertained to [litter size ≥ 12] according to the marker(s) in logistic regression. (23, 30-32). To predict the litter size using ROC curves, data were analyzed by Student's two-tailed *t*-test. Differences between high and low-litter size groups were considered significant at p values less than 0.05. Data are presented as mean ± SEM.

Results

Correlation of conventional semen parameters, sperm capacitation status, and expression of PRDXs with selected high- and low boar litter size

To find a sensitive marker for litter-size prediction, conventional semen analysis (including sperm motility, motion kinematics, and capacitation status analyses) and analysis of the mRNA levels of *PRDXs* were performed and results were compared between the high- (n=5) and low- litter size (n=5) groups. Our results showed that among *PRDXs*, only *PRDX 4* had significantly higher expression in the high-litter size group (Table 1, Figure 1B, $p < 0.05$). However, no significant association was found for other parameters between the two groups (Table 1).

Correlation of conventional semen parameters, sperm capacitation status, and PRDX expression levels with 20 boars with unknown litter sizes

Next, Pearson correlation analysis was conducted to demonstrate the correlation between different parameters and individual boars (n=20) with different litter sizes for increasing the accuracy of prediction. Only the mRNA levels of *PRDX 4* positively correlated with the litter size (R = 0.603, $p < 0.05$, Table 2). Although a significant correlation was observed between *PRDXs*, there was no correlation between the levels of *PRDXs* and the litter size, except for the level of *PRDX 4* ($p < 0.05$, Table 2). In addition, when sperm parameters were compared to the litter size, only HYP spermatozoa showed a significantly positive correlation with the litter size (R = 0.506, $p < 0.05$) (Table 2). Simultaneously, we performed western blotting under reducing (total Prdx 4) and non-reducing (thiol-oxidized Prdx 4) conditions to analyze the thiol-oxidation ratio of Prdx 4. However, we did not see any band for the non-reducing condition (thiol-oxidized Prdx4). This is seen as Prdx4 from all samples had already been oxidized by excessive levels of ROS, as shown in a

previously published study(16). Therefore, we analyzed the correlation between Prdx4 protein levels and individual boar samples (n=20) with different litter-sizes under reducing condition. Interestingly, the protein levels of Prdx 4 showed a significant negative correlation with the litter size, in contrast with the mRNA levels of *PRDX 4* (Figure 2, Table 2, R = -0.539, $p < 0.0$

Quality estimation of single/multiple markers for predicting litter size

For the screening test, we analyzed the following five key parameters: sensitivity, specificity, NPV, PPV, and overall accuracy. Before performing the quality assessment, we set the cut-off values for mRNA and protein levels of Prdx 4 and HYP as 0.71, 2.2939, and 11.82, respectively, based on the ROC curves (Table 4). Subsequently, to predict the average litter size, we used the cut-off values of mRNA and protein levels of PRDX 4 and HYP. Figure 1D shows that the ability of *PRDX 4* mRNA levels to predict high-litter size facilitated increasing the average litter size significantly (from 11.38 to 12.93) ($p < 0.05$). In addition, selecting boars with predicted high litter size based on HYP marker significantly increased the average litter size (from 11.94 to 12.87) (Figure 1C, $p < 0.01$). In contrast, the ability of Prdx 4 protein levels to predict low-litter size facilitated increasing the average litter size significantly (from 12.09 to 13).

Even though there was no correlation between the litter size and the levels of *PRDX* isoforms, except for the level of *PRDX 4*, we tried to discover markers that are more sensitive by combining *PRDX* isoforms with the protein levels of Prdx 4 to increase diagnostic accuracy. Backward multiple linear regression was performed using the, HYP level, mRNA expression levels of all PRDXs, and protein levels of Prdx 4; six types of models were found to be significantly related to the litter size ($p < 0.05$, Table 4). Subsequently, quality estimations (sensitivity, specificity, NPV, PPV, and overall accuracy) were conducted. Although the six types of models have much higher diagnostic accuracy compared to HYP and Prdx 4 protein levels, none of the combination models had higher diagnostic accuracy than the mRNA levels of *PRDX 4* (Table 4).

Discussion

Male infertility in both humans and animals is a major global problem that can lead to huge economic losses (29, 33). Many studies have been conducted to overcome male infertility, and 30-80% of infertile men exhibit spermatozoa damaged by ROS, which might be one of the cause of male infertility(1, 34, 35). Thus, to increase the accuracy of predicting male fertility, several studies have been conducted to discover an efficient antioxidant enzyme as a fertility-related biomarker(29, 36-38). The use of such biomarkers has greatly improved the diagnostic accuracy of male fertility. Therefore, we investigated whether the expression levels of *PRDXs* could be utilized commercially along with conventional semen analysis for predicting male fertility.

First, we investigated whether the litter size can be determined by conventional semen analysis. Our results showed that there was no significant difference between the high- (average litter size = 13.62 ± 0.25) and low-litter size group (average litter size = 11.34 ± 0.32) in terms of conventional semen parameters, such as sperm motility, motion kinematics, and capacitation status. These results corroborated those of Kwon et al. and further supported the limitation of conventional semen analysis for diagnostic accuracy(29). We then investigated the expression levels of *PRDX* as a potential biomarker for diagnosing male fertility in both high- and low-litter size groups.

PRDXs are among the most extensively studied antioxidant enzymes that counter reactive oxygen species by reducing hydrogen peroxide(2, 14-17, 39-41). It has been reported that Prdx 4 is associated with sperm acrosome formation and damage to sperm DNA(42). Furthermore, recently, it has been shown that the lack of Prdx 6 has an association with increased DNA fragmentation and oxidation.(13, 15, 43, 44). In addition, van Gestel et al. reported that Prdx 5 might have an indirect role in zona binding (45). Despite the fact that various isoforms of Prdx seem to affect spermatozoa, our results showed that out of the six isoforms, only *PRDX 4* is highly expressed in the high-litter size group. Based on this finding, we speculated that each isoform should be examined individually in order to identify the potential of different isoforms as biomarkers for a given species. Simultaneously, to gain further insight regarding correlation between Prdx 4 protein levels and the litter size, western blotting was conducted.

Correlation analysis was performed for each parameter, using the clinical fertility data of 20 randomly selected Yorkshire boars. Corroborating with the above result, among the six isoforms of *PRDXs*, only *PRDX 4* exhibited a significant positive correlation with litter size (r = 0.603). In contrast to *PRDX 4* mRNA levels, total Prdx 4 protein levels negatively correlated with the litter size (r = -0.539). This inverse pattern between mRNA and protein levels of *PRDX 4* might be explained by several factors, including regulation of translation and protein stability(46-48). Although difficult to explain, understanding the basis of this inverse correlation will be critical to explaining the relevance of *PRDX 4* transcript and protein markers. Our proteomic results corroborated those of Gong et al., who found that oxidized Prdx 1 and 6 are highly expressed in infertile men(13). Although oxidized bands could not be quantified, this could be explained to be a result of increased Prdx 4 di-sulfide bond formation due to high ROS levels as were found in O'Flaherty's study(16). This consensus in results might be due to the highly qualified redox ability of Prdx 4. Previous studies had reported that after losing its redox activity, Prdx 4 disrupts sperm physiology and function, resulting in cell death(49, 50). Although the biochemical significance of Prdx 4 in male fertility is not completely elucidated, it is tempting to speculate that Prdx 4 plays a considerable role in sperm quality and male fertility in swine compared to those in other mammals.

In contrast to several studies that found that inactive Prdxs led to loss of sperm motility and motion kinematics(2, 15), we found that Prdxs did not affect sperm motility and other motion parameters. This might suggest that the correlation between Prdxs and litter size is directly related through mechanisms other than reduction of sperm motility. Although there is no significant difference in HYP between high- and low-litter size groups, we observed that HYP also had a significant positive correlation with the litter size (r = 0.506). Evaluation of HYP is considered a powerful tool in

conventional semen analysis. To penetrate the zona pellucida of oocytes and execute successful fertilization, spermatozoa must acquire a special movement called HYP(51). Even though the Pearson correlation coefficient of HYP is relatively lower than that of Prdx 4, HYP might still be a representative biomarker among conventional semen parameters. Although HYP is a phenomenon that occurs after capacitation in the uterus, it is difficult to conclude whether it is pre-capacitated, as our results showed no alteration of capacitation status and acrosome reaction status. However, this is also a very interesting result and exploration of its implications requires further research.

Next, we performed quality assessment to determine the accuracy of these biomarkers using ROC curves. Our results revealed that the mRNA levels of *PRDX 4* are a superior male fertility biomarker compared to HYP and PRDX4 protein levels. All the evaluation indices showed that mRNA levels of *PRDX 4* exhibited higher accuracy than HYP and protein levels of Prdx 4 [Sensitivity: 93.75, 75, and 50 %, respectively; Specificity: 100, 75, and 50 %, respectively; NPV: 100, 42.56, and 20 %, respectively; PPV: 93.3, 92.31, 80 %, respectively; and Overall accuracy: 95, 75, and 50 %, respectively]. In addition, multiple linear regression analysis was performed with HYP, protein levels of Prdx 4, and the mRNA expression levels of all isoforms of *PRDXs* to increase the diagnostic accuracy of male fertility. However, none of the combination models showed a higher diagnostic accuracy than the mRNA levels of *PRDX 4*. Eventually, we evaluated the accuracy of this biomarker to predict litter size of field fertility data from 20 randomly selected pigs. As a biomarker to predict or diagnose the male fertility, the mRNA levels of *PRDX 4* were associated with a tremendous increase in the litter size (1.55 litter size/insemination). However, HYP and Prdx 4 protein expression were only associated with a slight increase in the litter size (0.93 and 0.91 litter size/insemination).

Conclusions

To the best of our knowledge, this is the first study to comprehensively elucidate the role of *PRDX 4* in male fertility. Our study demonstrated that *PRDX 4* plays the most significant role in male fertility and is the most sensitive biomarker for the prediction of male fertility in swine. Therefore, we speculated that *PRDX 4* has a good potential as a potential biomarker to increase litter size in swine. However, our research may have two limitations. i) Although several studies demonstrated that Prdxs are closely related with male fertility (15, 44, 52, 53), our study showed that other isotypes of *PRDX* did not exhibit any correlation with male fertility. For other *PRDX* isotypes, the correlation with a wider range of litter sizes may have been significant; however, no significant correlation was observed for the narrow range of litter size (10.3–14.2) used in the current study. ii) Even though each isoform of Prdxs is functional and can be interactive, we did not analyze all isoforms of Prdx at the protein level. Recent studies reported that lack of Prdx 6 in mice affected male fertility, reduced the efficacy of *in vitro* fertilization, and blocked development of early embryos before the blastocyst stage. Therefore, further studies should be conducted using large-scale field trials to identify whether the protein levels of other isoforms of Prdx are associated with the litter size, in order to identify more sensitive and accurate markers to predict male fertility.

Declarations

Author contributions: D.Y.R., W.K.P., M.S.R., and Y.J.P. performed the experiments. D.Y.R. and M.G.P. analyzed the data and created the artwork. D.Y.R., M.S.R., and Y.J.P. drafted the manuscript. M.G.P. supervised the design of the study and critically reviewed the manuscript. All authors critically reviewed the manuscript for intellectual content and gave final approval for the manuscript to be published.

Funding: This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET) through the Agri-Bio Industry Technology Development Program, funded by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA) (116172-3). Do-Yeal Ryu was supported by a NRF (National Research Foundation of Korea) grant funded by the Korean Government (NRF-2016-Fostering Core Leaders of the Future Basic Science Program/Global Ph.D. Fellowship Program).

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics declarations: All procedures were conducted according to the ethical guidelines for treatment of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University, Seoul, Republic of Korea (approval number: 2017-00018).

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests.

References

1. Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility. Nat Rev Urol. 2017;14(8):470-85.
2. Ryu DY, Kim KU, Kwon WS, Rahman MS, Khatun A, Pang MG. Peroxiredoxin activity is a major landmark of male fertility. Sci Rep. 2017;7(1):17174.
3. Fujii J, Ikeda Y, Kurahashi T, Homma T. Physiological and pathological views of peroxiredoxin 4. Free Radic Biol Med. 2015;83:373-9.
4. Walczak-Jedrzejowska R, Wolski JK, Slowikowska-Hilczer J. The role of oxidative stress and antioxidants in male fertility. Cent European J Urol. 2013;66(1):60-7.
5. Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ, Jr., et al. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod. 2004;19(1):129-38.

6. Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol. 2008;59(1):2-11.
7. Sabeti P, Pourmasumi S, Rahiminia T, Akyash F, Talebi AR. Etiologies of sperm oxidative stress. Int J Reprod Biomed (Yazd). 2016;14(4):231-40.
8. Dandekar SP, Nadkarni GD, Kulkarni VS, Punekar S. Lipid peroxidation and antioxidant enzymes in male infertility. J Postgrad Med. 2002;48(3):186-89; discussion 9-90.
9. Sabouhi S, Salehi Z, Bahadori MH, Mahdavi M. Human catalase gene polymorphism (CAT C-262T) and risk of male infertility. Andrologia. 2015;47(1):97-101.
10. Kawakami E, Takemura A, Sakuma M, Takano M, Hirano T, Hori T, et al. Superoxide dismutase and catalase activities in the seminal plasma of normozoospermic and asthenozoospermic Beagles. J Vet Med Sci. 2007;69(2):133-6.
11. Mahanta R, Gogoi A, Chaudhury PN, Roy S, Bhattacharyya IK, Sharma P. Association of oxidative stress biomarkers and antioxidant enzymatic activity in male infertility of north-East India. J Obstet Gynaecol India. 2012;62(5):546-50.
12. Lewis SE, Boyle PM, McKinney KA, Young IS, Thompson W. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertil Steril. 1995;64(4):868-70.
13. Gong S, San Gabriel MC, Zini A, Chan P, O'Flaherty C. Low amounts and high thiol oxidation of peroxiredoxins in spermatozoa from infertile men. J Androl. 2012;33(6):1342-51.
14. Kwon WS, Rahman MS, Lee JS, Kim J, Yoon SJ, Park YJ, et al. A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics. 2014;15:897.
15. Ozkosem B, Feinstein SI, Fisher AB, O'Flaherty C. Absence of Peroxiredoxin 6 Amplifies the Effect of Oxidant Stress on Mobility and SCSA/CMA3 Defined Chromatin Quality and Impairs Fertilizing Ability of Mouse Spermatozoa. Biol Reprod. 2016;94(3):68.
16. O'Flaherty C, de Souza AR. Hydrogen peroxide modifies human sperm peroxiredoxins in a dose-dependent manner. Biol Reprod. 2011;84(2):238-47.
17. Sutovsky P, Aarabi M, Miranda-Vizuete A, Oko R. Negative biomarker based male fertility evaluation: Sperm phenotypes associated with molecular-level anomalies. Asian J Androl. 2015;17(4):554-60.
18. Lunney JK. Advances in swine biomedical model genomics. Int J Biol Sci. 2007;3(3):179-84.
19. Broekhuijse ML, Feitsma H, Gadella BM. Artificial insemination in pigs: predicting male fertility. Vet Q. 2012;32(3-4):151-7.
20. Roehe R, Kennedy BW. Estimation of genetic parameters for litter size in Canadian Yorkshire and Landrace swine with each parity of farrowing treated as a different trait. J Anim Sci. 1995;73(10):2959-70.
21. Humphray SJ, Scott CE, Clark R, Marron B, Bender C, Camm N, et al. A high utility integrated map of the pig genome. Genome Biol. 2007;8(7):R139.
22. Kwon WS, Rahman MS, Ryu DY, Khatun A, Pang MG. Comparison of markers predicting litter size in different pig breeds. Andrology. 2017;5(3):568-77.
23. Kim KU, Pang WK, Kang S, Ryu DY, Song WH, Rahman MS, et al. Sperm solute carrier family 9 regulator 1 is correlated with boar fertility. Theriogenology. 2019;126:254-60.
24. Shukla KK, Kwon WS, Rahman MS, Park YJ, You YA, Pang MG. Nutlin-3a decreases male fertility via UQCRC2. PLoS One. 2013;8(10):e76959.
25. Mortimer ST, Swan MA, Mortimer D. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. Hum Reprod. 1998;13(8):2139-46.
26. Rahman MS, Kwon WS, Karmakar PC, Yoon SJ, Ryu BY, Pang MG. Gestational Exposure to Bisphenol A Affects the Function and Proteome Profile of F1 Spermatozoa in Adult Mice. Environ Health Perspect. 2017;125(2):238-45.
27. Kwon WS, Shin DH, Ryu DY, Khatun A, Rahman MS, Pang MG. Applications of capacitation status for litter size enhancement in various pig breeds. Asian-Australas J Anim Sci. 2018;31(6):842-50.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
29. Kwon WS, Rahman MS, Lee JS, Yoon SJ, Park YJ, Pang MG. Discovery of predictive biomarkers for litter size in boar spermatozoa. Mol Cell Proteomics. 2015;14(5):1230-40.
30. Kang S, Pang WK, Ryu DY, Song WH, Rahman MS, Park YJ, et al. Porcine seminal protein-I and II mRNA expression in boar spermatozoa is significantly correlated with fertility. Theriogenology. 2019;138:31-8.
31. Oh SA, Park YJ, You YA, Mohamed EA, Pang MG. Capacitation status of stored boar spermatozoa is related to litter size of sows. Anim Reprod Sci. 2010;121(1-2):131-8.
32. Kwon WS, Rahman MS, Ryu DY, Park YJ, Pang MG. Increased male fertility using fertility-related biomarkers. Sci Rep. 2015;5:15654.
33. Inhorn MC, Patrizio P. Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. Hum Reprod Update. 2015;21(4):411-26.
34. Aitken J, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioessays. 1994;16(4):259-67.
35. Agarwal A, Prabakaran S, Allamaneni S. What an andrologist/urologist should know about free radicals and why. Urology. 2006;67(1):2-8.
36. Wang J, Wang J, Zhang HR, Shi HJ, Ma D, Zhao HX, et al. Proteomic analysis of seminal plasma from asthenozoospermia patients reveals proteins that affect oxidative stress responses and semen quality. Asian J Androl. 2009;11(4):484-91.

37. Bieniek JM, Drabovich AP, Lo KC. Seminal biomarkers for the evaluation of male infertility. Asian J Androl. 2016;18(3):426-33.
38. Platts AE, Dix DJ, Chemes HE, Thompson KE, Goodrich R, Rockett JC, et al. Success and failure in human spermatogenesis as revealed by teratozoospermic RNAs. Hum Mol Genet. 2007;16(7):763-73.
39. Awad MM, Graham JK. A new pellet technique for cryopreserving ram and bull spermatozoa using the cold surface of cattle fat. Anim Reprod Sci. 2004;84(1-2):83-92.
40. Anwar S, Yanai T, Sakai H. Overexpression of Peroxiredoxin 6 Protects Neoplastic Cells against Apoptosis in Canine Haemangiosarcoma. J Comp Pathol. 2016;155(1):29-39.
41. Goemaere J, Knoops B. Peroxiredoxin distribution in the mouse brain with emphasis on neuronal populations affected in neurodegenerative disorders. J Comp Neurol. 2012;520(2):258-80.
42. Sasagawa I, Matsuki S, Suzuki Y, Iuchi Y, Tohya K, Kimura M, et al. Possible involvement of the membrane-bound form of peroxiredoxin 4 in acrosome formation during spermiogenesis of rats. Eur J Biochem. 2001;268(10):3053-61.
43. O'Flaherty C. Orchestrating the antioxidant defenses in the epididymis. Andrology. 2019.
44. Ozkosem B, Feinstein SI, Fisher AB, O'Flaherty C. Advancing age increases sperm chromatin damage and impairs fertility in peroxiredoxin 6 null mice. Redox Biol. 2015;5:15-23.
45. van Gestel RA, Brewis IA, Ashton PR, Brouwers JF, Gadella BM. Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of the oocyte. Mol Hum Reprod. 2007;13(7):445-54.
46. Schwanhausser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. Nature. 2011;473(7347):337-42.
47. Jovanovic M, Rooney MS, Mertins P, Przybylski D, Chevrier N, Satija R, et al. Immunogenetics. Dynamic profiling of the protein life cycle in response to pathogens. Science. 2015;347(6226):1259038.
48. Moritz CP, Muhlhaus T, Tenzer S, Schulenborg T, Friauf E. Poor transcript-protein correlation in the brain: negatively correlating gene products reveal neuronal polarity as a potential cause. J Neurochem. 2019;149(5):582-604.
49. Iuchi Y, Okada F, Tsunoda S, Kibe N, Shirasawa N, Ikawa M, et al. Peroxiredoxin 4 knockout results in elevated spermatogenic cell death via oxidative stress. Biochem J. 2009;419(1):149-58.
50. Lee D, Moawad AR, Morielli T, Fernandez MC, O'Flaherty C. Peroxiredoxins prevent oxidative stress during human sperm capacitation. Mol Hum Reprod. 2017;23(2):106-15.
51. Suarez SS, Ho HC. Hyperactivated motility in sperm. Reprod Domest Anim. 2003;38(2):119-24.
52. Liu Y, O'Flaherty C. In vivo oxidative stress alters thiol redox status of peroxiredoxin 1 and 6 and impairs rat sperm quality. Asian J Androl. 2017;19(1):73-9.
53. O'Flaherty C. Peroxiredoxin 6: The Protector of Male Fertility. Antioxidants (Basel). 2018;7(12).

Tables

Table 1. Difference in sperm motility, motion kinematics, capacitation status, and expression levels of peroxiredoxins (*PRDXs*) between high- and low-litter size groups.

Parameters			High-litter size group (n=5)	Low-litter size group (n=5)	*P* value
Sperm motility and	motion kinematics	MOT (%)	79.19 ± 5.10	77.61 ± 4.25	0.817
		HYP (%)	17.73 ± 3.74	10.99 ± 1.70	0.139
		VCL (μm/sec)	143.39 ± 9.37	135.22 ± 6392	0.503
		VSL (μm/sec)	63.60 ± 3.77	65.66 ± 6.96	0.801
		VAP (μm/sec)	74.40 ± 4.13	74.70 ± 6.40	0.970
		LIN (%)	44.46 ± 1.14	48.24 ± 3.44	0.327
		BCF (Hz)	11.76 ± 0.36	12.23 ± 0.44	0.427
		WOB (%)	51.97 ± 0.80	55.04 ± 2.90	0.336
		ALH (μm/sec)	6.27 ± 0.39	6.10 ± 0.36	0.764
Capacitation status		AR (%)	0.52 ± 0.39	2.77 ± 1.97	0.295
		B (%)	8.24 ± 2.45	8.45 ± 3.13	0.675
		F (%)	91.24 ± 2.79	88.78 ± 4.94	0.958
Gene expression		*PRDX 1*	1.00 ± 0.18	0.97 ± 0.05	0.892
		PRDX 2	1.11 ± 0.19	1.39 ± 0.14	0.273
		PRDX 3	0.95 ± 0.08	1.13 ± 0.08	0.142
		PRDX 4	0.88 ± 0.05	0.69 ± 0.03	0.011
		PRDX 5	0.74 ± 0.06	0.65 ± 0.09	0.445
		PRDX 6	0.89 ± 0.19	0.91 ± 0.12	0.931

MOT = motility; HYP = hyperactivation; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity; BCF = beat cross frequency; WOB = wobble; ALH = mean amplitude of head lateral displacement; AR = acrosome-reacted spermatozoa; B = capacitated spermatozoa; F = non-capacitated spermatozoa; PRDX = peroxiredoxin. Replication (n = 5), data represent mean of five replicates ± SEM. ($p < 0.05$, calculated using two-tailed Student's *t*-test).

Table 2. Correlation analysis for sperm motility, motion kinematics, capacitation status, gene expression, Prdx 4 protein expression, and litter size of 20 Yorkshire boars.

	Sperm motility and motion kinematics									Capacitation status			Gene expression						Prdx4 protein	LITTER SIZE
	MOT	HYP	VCL	VSL	VAP	LIN	BCF	WOB	ALH	AR	F	B	*PRDX 1*	*PRDX 2*	*PRDX 3*	*PRDX 4*	*PRDX 5*	*PRDX 6*		
MOT	1	.713**	.836**	0.388	.575**	-0.134	-.778**	-0.112	.788**	-0.162	0.265	-0.277	-0.067	-0.056	-0.036	0.391	-0.041	-0.098	-0.174	0.207
HYP		1	.842**	0.188	0.387	-0.39	-.769**	-0.418	.707**	-0.047	0.079	-0.083	0.264	0.287	0.313	.463*	0.269	0.261	-0.374	.506*
VCL			1	.644**	.793**	0.075	-.898**	0.038	.969**	-0.214	0.215	-0.173	0.070	0.041	0.052	0.341	0.038	0.079	-0.201	0.338
VSL				1	.963**	.810**	-.551*	.764**	.793**	-0.373	0.213	-0.069	-0.097	-0.197	-0.198	-0.124	-0.154	-0.012	-0.014	-0.026
VAP					1	.650**	-.683**	.637**	.907**	-0.361	0.266	-0.152	-0.079	-0.151	-0.154	-0.014	-0.125	-0.045	-0.059	0.016
LIN						1	-0.022	.972**	0.291	-0.351	0.138	0.025	-0.161	-0.270	-0.280	-0.415	-0.203	-0.062	0.108	-0.274
BCF							1	0.026	-.858**	0.195	-0.193	0.152	-0.017	-0.068	-0.100	-0.351	0.002	-0.005	0.129	-0.360
WOB								1	0.262	-0.351	0.184	-0.041	-0.206	-0.290	-0.311	-0.437	-0.234	-0.158	0.127	-0.378
ALH									1	-0.226	0.207	-0.153	0.001	-0.045	-0.036	0.221	-0.049	0.030	-0.122	0.197
AR										1	-.803**	.517*	-0.272	-0.294	-0.274	-0.066	-0.361	-0.104	0.245	-0.275
F											1	-.926**	0.271	0.361	0.327	0.157	0.304	-0.064	-0.135	0.128
B												1	-0.216	-0.332	-0.295	-0.183	-0.208	0.158	0.038	-0.009
PRDX 1													1	.876**	.898**	-0.219	.903**	.831**	-0.296	0.222
PRDX 2														1	.976**	-0.033	.926**	.636**	-0.287	0.299
PRDX 3															1	-0.057	.920**	.695**	-0.347	0.285
PRDX 4																1	-0.182	-0.247	-0.037	.603**
PRDX 5																	1	.745**	-.485*	0.323
PRDX 6																		1	-0.442	0.303
Prdx 4 protein																				-.539*
LITTER SIZE																				1

MOT = motility; HYP = hyperactivation; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity; BCF = beat cross frequency; WOB = wobble; ALH = mean amplitude of head lateral displacement; AR = acrosome-reacted spermatozoa; B = capacitated spermatozoa; F = non-capacitated spermatozoa; PRDX = peroxiredoxin. Number of animals = 20. *$p < 0.05$, **$p < 0.01$, calculated using Pearson correlation coefficients.

Table 3. Multiple marker analyses using seven markers via backward multiple regression.

Models	Markers	UMA	
		Standardized β Value	*P*value
Y1	HYP	-0.018	0.023
	PRDX 1	0.025	
	PRDX 2	0.713	
	PRDX 3	-0.921	
	PRDX 4	0.688	
	PRDX 5	0.175	
	PRDX 6	0.338	
	Prdx 4 (Protein)	-0.393	
Y2	HYP	-0.016	0.010
	PRDX 2	0.708	
	PRDX 3	-0.909	
	PRDX 4	0.687	
	PRDX 5	0.187	
	PRDX 6	0.347	
	Prdx 4 (Protein)	-0.388	
Y3	*PRDX 2*	0.712	0.004
	PRDX 3	-0.915	
	PRDX 4	0.679	
	PRDX 5	0.188	
	PRDX 6	0.344	
	Prdx 4 (Protein)	-0.384	
Y4	*PRDX 2*	0.901	0.001
	PRDX 3	-0.962	
	PRDX 4	0.653	
	PRDX 6	0.370	
	Prdx 4 (Protein)	-0.426	
Y5	*PRDX 2*	0.038	0.001
	PRDX 4	0.657	
	PRDX 6	0.271	
	Prdx 4 (Protein)	-0.384	
Y6	*PRDX 4*	0.662	< 0.001
	PRDX 6	0.297	
	Prdx 4 (Protein)	-0.383	

PRDX: peroxiredoxin. Y1) HYP, mRNA levels of *PRDX 1 to 6*, and protein levels of Prdx 4. Y2) HYP, mRNA levels of *PRDX 2 to 6*, and protein levels of Prdx 4. Y3) mRNA levels of *PRDX 2 to 6* and protein levels of Prdx 4. Y4) mRNA levels of *PRDX 2 to 4 and 6* and protein levels of Prdx 4. Y5) mRNA levels of *PRDX 2, 4, and 6* and protein levels of Prdx 4. Y6) mRNA levels of *PRDX 4, and 6* and protein levels of Prdx 4.

Table 4. Quality assessment of levels of PRDX 4 expression (both mRNA and protein levels), hyperactivation (HYP), and marker combination models according to the receiver-operating characteristic curves.

	Cut-off value	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Positive predictive value (%)	Overall accuracy (%)
PRDX 4 expression	0.71	93.75	100	100	93.3	95
HYP	11.82	75	75	42.86	92.31	75
Prdx 4 protein	2.2939	50	50	20	80	50
Y1	12.2813	75	100	100	100	80
Y2	12.1867	81.25	100	100	100	85
Y3	12.2861	75	100	100	100	80
Y4	12.1669	81.25	100	100	100	85
Y5	12.0969	81.25	100	100	100	85
Y6	12.0993	81.25	100	100	100	85

All cut-off value was decided by the predicted probability that the individual semen appertained to [litter size ≥ 12] according to the marker(s) in logistic regression. Sensitivity: according to the litter size, boars showing true positive results are expressed as a percentage of boars based on the test. Specificity: percentage of boars evaluated as truly negative. Positive predictive value: rate of boars evaluated as positive but truly having a litter size ≥12 or <12. Negative predictive value: rate of boars that demonstrated negative correlation, but had an actual litter size of ≥12 or <12 (based on the average of Yorkshire boar litter size). PRDX: peroxiredoxin. Y1) HYP, mRNA levels of *PRDX 1 to 6* and protein levels of Prdx 4. Y2) HYP, mRNA levels of *PRDX 2 to 6* and

protein levels of Prdx 4. Y3) mRNA levels of *PRDX 2 to 6* and protein levels of Prdx 4. Y4) mRNA levels of *PRDX 2 to 4 and 6* and protein levels of Prdx 4. Y5) mRNA levels of *PRDX 2, 4, and 6* and protein levels of Prdx 4. Y6) mRNA levels of *PRDX 4 and 6* and protein levels of Prdx 4.

Figures

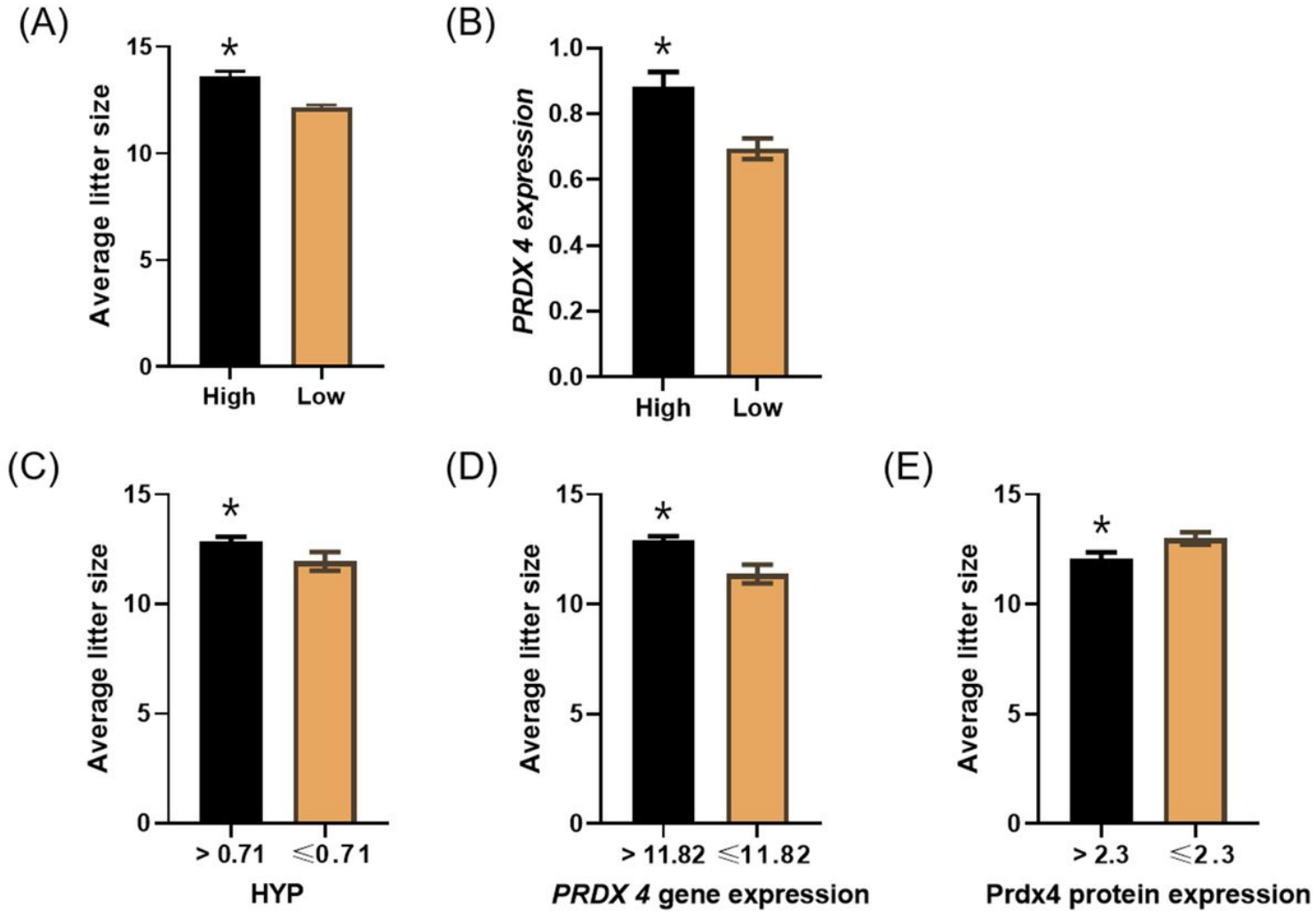

Figure 1

Difference in parameters between high- and low-litter size groups and difference in average litter size based on hyperactivation and mRNA levels of peroxiredoxin 4 (PRDX 4) (A) Difference in average litter size between high- and low-litter size groups (n = 5). (B) Difference in the levels of PRDX 4 expression between high- and low-litter size groups (n = 5). Data represent the mean of five replicates ± SEM. (C) Difference in average litter size according to hyperactivation (>11.82; n = 13, ≤11.82; n = 7). (D) Difference in average litter size according to mRNA levels of PRDX 4 (>0.71; n = 15, ≤0.71; n = 5). (E) Difference in average litter size according to protein levels of Prdx 4 (>2.2939; n=10, ≤ 2.2939; n=10) ($p < 0.05$, calculated using two-tailed Student's t-test).

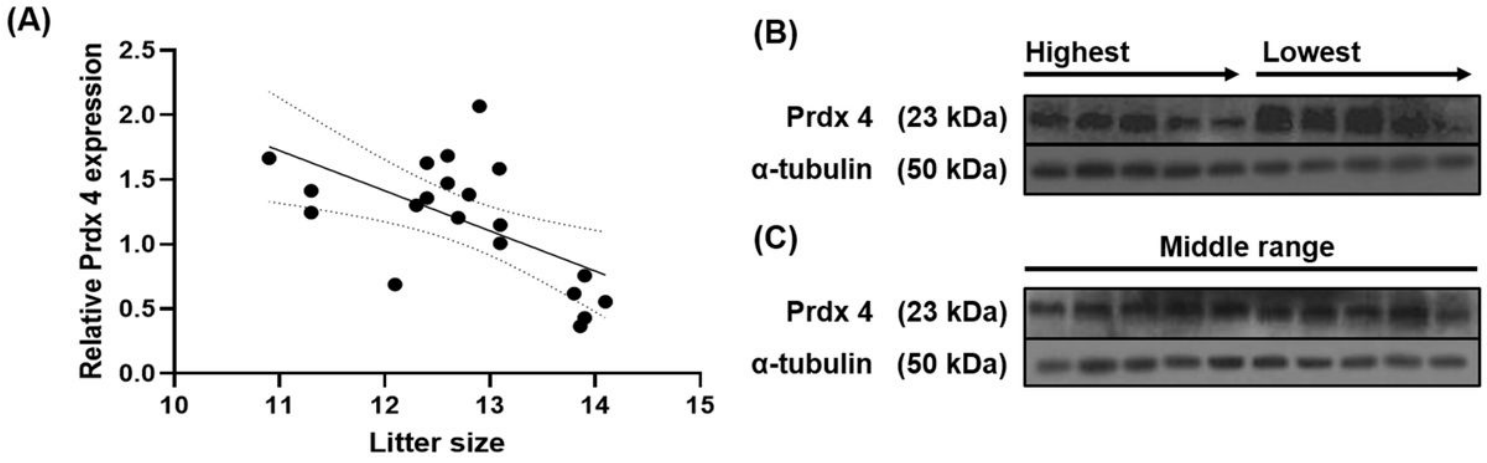

Figure 2

Prdx 4 expression in spermatozoa from individual boar with different litter size. (A) Correlation between litter size and relative Prdx 4 expression. (B) Representative western blot of Prdx4 probed with anti-peroxiredoxin-4 antibody in ten boars with 5 higher and 5 lower litter size. (C) Representative western blot of Prdx4 probed with anti-peroxiredoxin-4 antibody in ten boars with middle range litter size