Oxidative stress and NRF2 gene expression in blood of indigenous pigs

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Abstract

The present study examined the variability of oxidative stress levels and NRF2 gene expression between blood samples of piglet and adult pigs of indigenous origin. Among various stressors, higher oxidative stress is one of the most important factors associated with multiple health and managemental problems commonly observed in piglets. NRF2 is a key regulator of transcriptional activation of antioxidant response genes and oxidative stress. Therefore, the present research work was conducted to study NRF2 gene expression in blood samples of indigenous piglets (N=8) and adult pigs (N=8) using de novo synthesized primers specific to Sus Scrofa species. Further, lipid peroxidation levels in RBC hemolysate samples were compared between piglets and adult pigs by measuring Malondialdehyde levels by thiobarbituric acid reactive substance (TBARS) assay. Association of NRF2 gene expression with MDA lipid peroxidation levels in indigenous pigs were calculated by Pearson's correlation method. In present study we found, significantly lower levels ($P < 0.01$) of NRF2 gene expression and higher levels ($P < 0.01$) of MDA lipid peroxidation in RBC hemolysate were present in piglets as compared to adult pigs. Further, MDA lipid peroxidation levels were found to significant ($P < 0.01$) negative correlation with NRF2 fold gene expression levels. Low levels of NRF2 expression adversely impacting piglet's health and makes them prone to higher oxidative stress. Present findings suggest that NRF2 gene expression levels are effective molecular markers for determining, modulating and improving the antioxidant capacity of indigenous piglets.

Key Words: NRF2 gene, gene expression, oxidative stress, indigenous pig

Introduction

Animal husbandry is a vital aspect of agriculture diversification, and plays a crucial role in increasing rural incomes, doubling farmers' earnings, and there is a need for higher public investment in the livestock sector. Pigs are reared by socio-economically weaker sections of the society and contribute 1.7% to the total livestock population (20th Livestock Census, 2019). Due to their high fecundity, better feed conversion efficiency, early maturity, and short generation interval, pig farming has high potential for providing faster economic returns with minimal investment in housing. Despite low profit, indigenous pig production provides livelihood for traditional pig breeders while utilizing available garbage and other wastes to convert them into valuable proteins (Behl *et al.*, 2020, Muhindro Singh 2023). Although, growth of indigenous pig rearing is slower than exotic pig, total returns per month cost of feeding per kg of live body weight and packaged pork in indigenous and exotic pigs revealed that indigenous pigs could make a significant contribution to the economy of the weaker section of society with meagre resources and they supply animal proteins at cheaper rates (Naskar et al., 2013).

Piglet mortality is one of the most common problems encountered in pig production (Ovcharova et al 2022). The small size of piglets at the time of birth, with their low body energy storage and their immature immune system, make them more prone to chilling, starving, or increased oxidative stress (Farmer and Edwards, 2022). Studies have also indicated that weaning disrupts the physiologic equilibrium of oxidants and antioxidants in the body, which leads to oxidative stress with eventually induces enterocyte apoptosis and cell cycle arrest in the small intestine of post weaning piglets (Zhu *et al.*, 2012, Zhu *et al.*,2013). The liver is a thermogenic organ that contains a large number of mitochondria in mammals, which is the place with high oxygen consumption and reactive oxygen species (ROS) formation (Assaad, 2014). Although, technological advancements have mitigated some of these negative consequences, a better understanding of the biological impact of stress is necessary to reduce weaning stress.

Oxidative stress refers to the dramatic increase in free radical production in the animal's body, breaking the antioxidation-oxidation balance or a decrease in the ability to scavenge free radicals (Baird and Yamamoto, 2020). Oxidative stress can result from internal metabolism and external exposure, leading to cellular dysfunction and contributing to chronic diseases such as diabetes, cancer, and neurodegenerative diseases (Hao et al., 2021). The oxidative status of pigs evolves from the neonatal period to puberty, with an increase in plasma concentration of hydroperoxides (HPO) and stabilization of antioxidant capacity (Buchet et al., 2017).

It remains unclear whether the observed oxidative stress is a result of the weaning process or other biological mechanisms related with environmental factors or maturation. The developmental trajectory of oxidative status during the growth phase of mammals has not been characterized.

Transcription factor, nuclear factor erythroid 2- related factor 2 (NRF2) is an emerging mediator of cellular defence against oxidative stress. It marks the expression of genes that contain antioxidant response elements, thereby regulating the physiological and pathophysiological consequences of exposure to oxidants (Hao et al., 2021). The role of NRF2 gene in resistance to oxidative stress is a critical function that has been revealed in recent studies.

The NRF2 gene is located on chromosome number 21 and is regulated by the transcription factor, Kelch-like ECH-associated protein 1 (KEAP1). Under normal conditions, KEAP1 targets NRF2 for degradation by the ubiquitin-proteasome system, maintaining low levels of NRF2. However, in response to oxidative stress, NRF2 undergoes stabilization and translocation to the nucleus, where it forms a heterodimer with small Maf proteins, binding to antioxidant response elements (AREs) in target gene promoters and inducing gene expression (Zhang et al, 2015). The nuclear factor erythroid 2-related factor 2 (NRF2) is a master regulator of redox homeostasis. NRF2 plays an important role for cellular protection by inducing the expression of genes related to antioxidant, anti-inflammatory, and cytoprotective response. (Bukke et al., 2022).

Overall, the impact of oxidative stress on pig performance is significant, making it an important cause for affecting pig production in India. Considering the regulation of antioxidant response by NRF2 and its role in cellular resistance to oxidative stress, NRF2 may be used as a potential marker or indicator of the antioxidant status of pigs. Thus, a study was planned to know the effects of NRF2 gene expression on oxidative stress in indigenous pigs.

Materıals and Methods

Location of the study area

The study was conducted at Department of Animal Genetics and Breeding, Veterinary Biochemistry, College Pig Farm in Livestock Farm Complex of Krantisinh Nana Patil College of Veterinary Science (KNPCVS), Shirwal (between latitude 18.13°N and longitude 73.98°E), Dist. - Satara, Maharashtra, India during September to March, 2023. The Shirwal has typical monsoon climate, with moderately hot, rainy and cold weather seasons. Summer season is predominantly observed during March, April and May (the hottest months) and temperature varies between 22°C-39°C during this season. Winter is cool, dry and pleasant weather prevails from October to February. Temperature varies in this season between 12°C-34°C while about 80-90% of the rainfall is concentrated

in the months of June to September. The average annual rainfall in this region is 650 mm (Ahire et.al., 2021). In Shirwal, the average percentage of the sky covered by clouds experiences extreme seasonal variation over the year.

Sample collection

Study was conducted on indigenous pigs maintained at KNP College of Veterinary Science Shirwal college pig farm, under ICAR-AICRP pig project sub centre of NRC on pig, Guwahati. These indigenous pigs were grouped in two categories based on their age as Group I adult indigenous pigs above 2 years of age (n=8) and Group II indigenous piglets of 4 to 6 months age (n=8). Blood sample of 5 ml was collected in heparin containing vials from pigs from the lateral saphenous vein using a sterile 22 G scalp vein. Out of this, 2 ml was transferred to a heparinized vial and used for hemolysate preparation for MDA assay and 2 ml of blood was used for extraction of total RNA and subsequent real-time PCR experiments in molecular study.

MDA (TBARS) Assay in RBC hemolysate

The reaction mixture for assay contained 1ml of 0.67% TBA, 0.5 ml TCA and 0.2 ml of RBC hemolysate (Total volume 1.7 ml). This was incubated or heated at 100^0C for 20 minutes. Control sample was not incubated or heated. Then, sample and control were centrifuged at 12,000 rpm for 5 minutes. Absorbance was recorded at 532 nm using UV/VIS spectrophotometer. All determinations were performed in duplicate. MDA concentration was determined by using a molar extinction co-efficient of 1.52X105 /M/cm at 532 nm. The values of MDA in the sample were expressed as nMol/g of Hb.

The measurement of Malondialdehyde (MDA), the decomposition products of oxidized polyunsaturated fatty acids, is commonly used as a method for the quantification of lipid peroxidation. The method is based on the reaction of MDAs with thiobarbituric acid, which forms a thiobarbituric acid reactive substances (TBARS) chromophore (Kanias *et al.*, 2007).

NRF2 gene expression study

Relative gene expression study of NRF2 gene in indigenous piglets and adult pigs was carried out by real time PCR using SYBR green based method. NRF2 gene is a transcription factor that controls the cellular defense against toxic and oxidative insults through the expression of genes involved in oxidative stress response and drug detoxification (Feng *et al.*, 2020).

Total RNA extraction and quality determination

Total RNA was isolated using trizol reagent (SRL). Purity of RNA was checked by using Thermo Nano-Drop spectrophotometer. The dissolved 2 µl RNA was added to find out the ratios of optical density (O.D.) at 260 nm and 280 nm. Quality of RNA was assessed by electrophoresis on a denaturing agarose (1.5% w/v gel). 30 ml of 1.5% agarose gel was used along with 4 µl Ethidium Bromide for staining of the bands. The RNA suspension was further processed for cDNA preparation.

Reverse Transcription and Quantitative Real-Time PCR

The 1 µg RNA was reversed transcribed to complementary DNA (cDNA) using cDNA synthesis kit (Fermentas) according to manufacture instructions. First strand of cDNA was confirmed by amplification of beta actin gene.

Primer Designing

The primers were designed by using the Real-time PCR Primer Design Tool from GenScript. The specificity of the primers was checked using BLAST from NCBI and details with primers has been presented in Table 1. Finally, the primers were ordered from Integrated DNA Technologies (IDT). Quantitative Real-time PCR (qPCR) was performed with Invitrogen Sybr green ®Supermix kit. The qPCR conditions were as follows given in Table 2. Mean ΔΔCt of NRF2 gene expression of indigenous piglets relative to the Adult indigenous pigs was compared.

Statistical Analysis

All the values were expressed as overall mean ± SEM. Statistical analysis of the experimental data was carried out according to the suitable statistical methods. Student's t-test was used to test whether the difference between the values of lipid peroxidation MDA levels and mRNA expressions of NRF2 gene in adult and piglet of two groups. Various parameters were determined by Karl Pearson's coefficient of correlation. Results were considered significant when $p<0.05$.

Results

MDA Assay

As per the results, values of MDA assay between adults and piglets differed significantly. Values of lipid peroxidation MDA levels were found significantly lower in adult as compared to piglets. Values of overall mean ± SE of MDA assay study has been represented in the table 3 and graphically represented in the fig 1.

Gene expression study

The mRNA expressions of NRF2 gene was found to be significantly lower (P <0.01) in piglets in relation to adult indigenous pigs. Gene expression fold (2^-ΔΔct) and log 2 fold relative mRNA expression in piglets relative to adult indigenous pigs of target gene viz NRF2 has been presented in Table 4 and also, Gene expression fold (2^-ΔΔct) and log2 fold relative mRNA expression in piglets relative to adult indigenous pigs of target gene viz NRF2 has been graphically represented in figure 2 and 3, respectively. Also, the qPCR data amplification plot and Melt Curve have been graphically represented from figures 4 to7 respectively.

Correlation of NRF2 gene with MDA lipid peroxidation

Correlation study of fold expression of NRF2 gene in adult indigenous pigs relative to piglet with MDA assay revealed a negative correlation (P <0.01) shown in Table 5.

Table 1: Primer sequences for Real-time PCR

S.N.	Name of the gene	Amplicon size (bp)	Primer Sequences	Primer length (bp)	Primer Tm
1	NRF2	183	**F:** CAGCAACAGCATGCCCTCCT	20	61.40
			R: GCCATGCTGGGACTTGGGTT	20	61.40
2	GAPDH	134	**F:** GACTGTGGATGGCCCGTCTG	20	63.45
			R: TGCCAGTGAGCTTCCCGTTG	20	61.40
3	ACTB	185	**F:** CGGGACCTGACCGACTACCT	20	63.45
			R: CGGGCAGCTCGTAGCTCTTC	20	63.45

Table 2: Cyclic condition used for Real- time PCR

Segment	Remarks	Thermal Profile (˚C)	Time	No of cycles
1	Initial Denaturation	95˚C	2 min	1
2	Denaturation	95˚C	10 sec	40
	Annealing	58˚C	30 Sec	
3	Melt Curve/Dissociation curve analysis	95˚C	1 min	1
		65˚C	30 sec	
		65˚C -95˚C	3 degree per min	
		95˚C	30 sec	

Table 3: Mean Lipid peroxidation level nMol/mg of Hb in Indigenous pigs

S. N.	Groups of pig	MDA nMol/g of Hb	t value	P value
1	Adult	1.193± 0.042	2.49	0.012*
2	Piglet	1.326 ±0.041		

Significant at *P<0.05

Table 4: Fold expression and log fold expression change of Sus Scrofa NRF2

S. N.	Groups	Fold expression	Log fold gene expression change
1	Adult indigenous pig	4.466 ± 0.970	3.387± 0.735
2	Indigenous Piglet	1.356 ±0.114	1.00 ± 0.090

Table 5: Bivariate Pearson correlation between MDA level and fold gene expression of NRF2.

S. N.	Groups	'r' value	'p' value
1	MDA	-0.635	0.006**
2	Fold expression NRF2		

Significant at **P<0.01

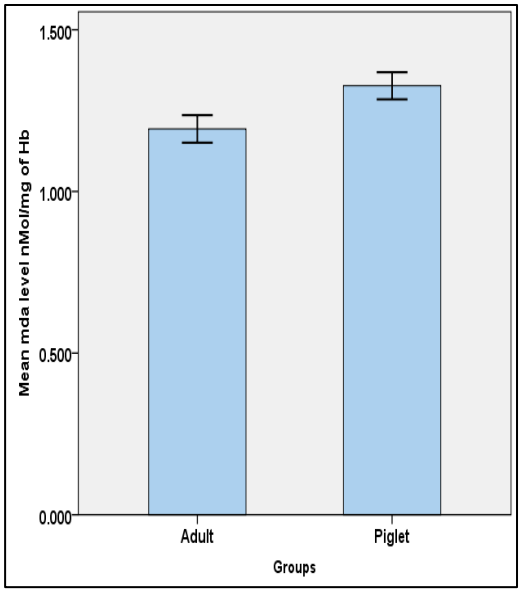

Fig 1 Lipid peroxidation MDA level nMol /mg of Hb in indigenous adult pigs and piglets

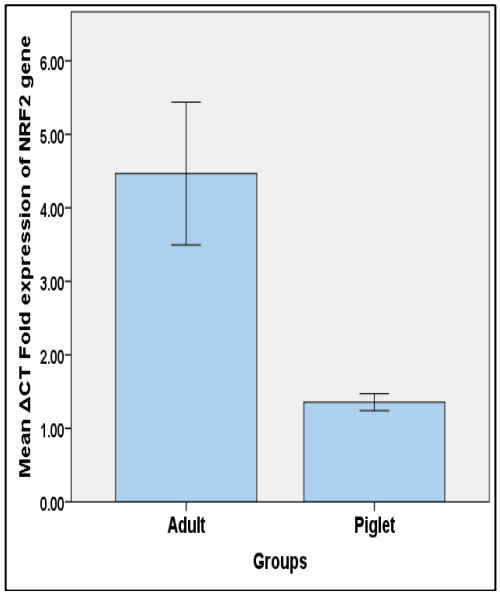

Fig 2 Mean fold expression of NRF2 gene in adult indigenous pigs and indigenous piglet

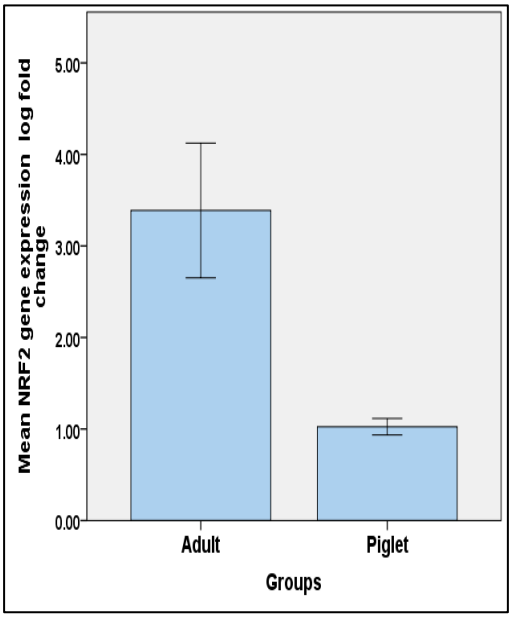

Fig 3 Mean NRF2 gene expression log fold change in adult indigenous pigs relative to indigenous piglet

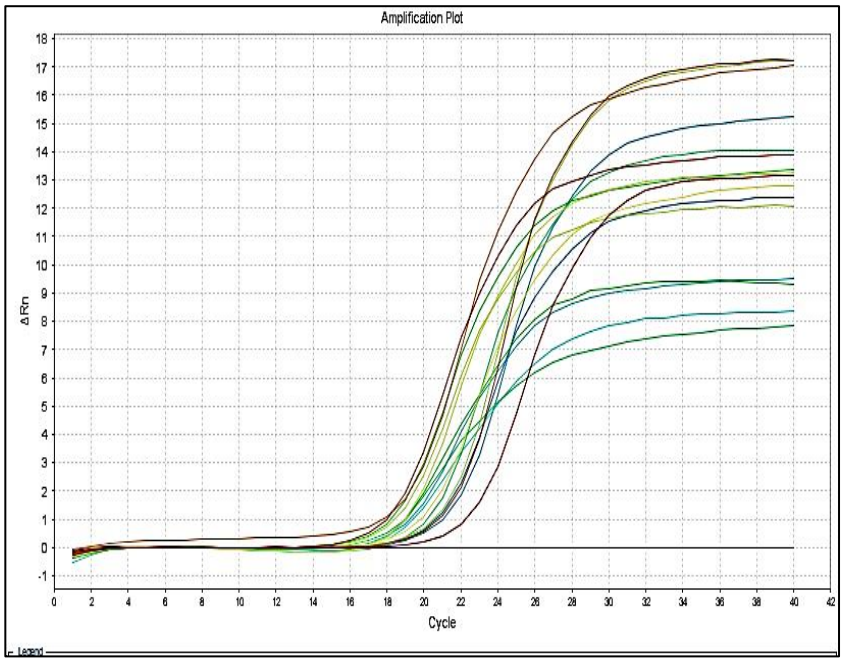

Fig 4 Amplification plot NRF2 gene

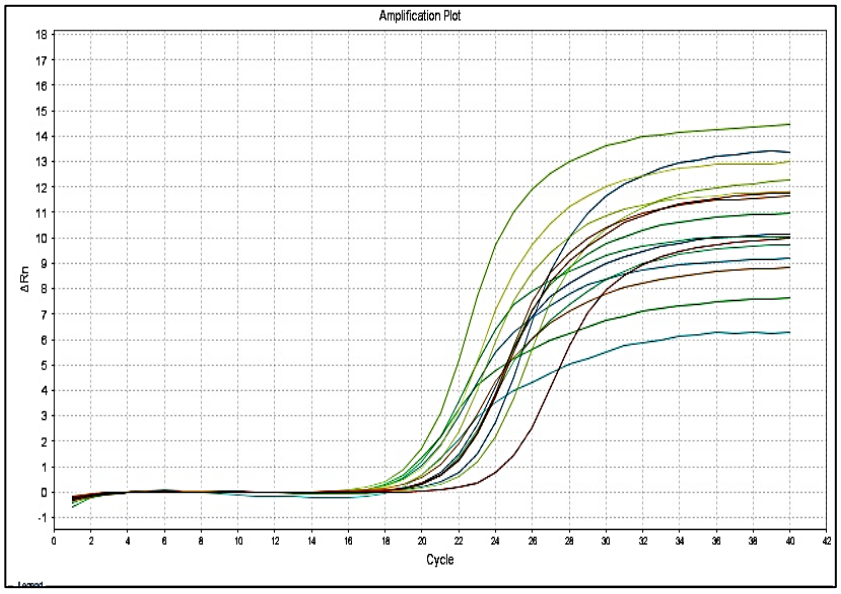

Fig 5 Amplification plot β-Actin gene

Discussion

Higher oxidative stress is one of the factors responsible for higher mortality observed in young piglets. Studies regarding correlation of genetic factors responsible for this observed poor antioxidant response found in piglets compared to adult pigs has not been studied previously. Therefore, study was planned to compare expression of NRF2 gene which is considered as direct regulator of antioxidant enzyme levels with oxidative stress levels in young piglets compared to adult indigenous pigs. The results of present study show higher MDA lipid peroxidation levels in the piglets as compared to adult pigs. It was found that the level of blood lipid oxidation product Malondialdehyde (MDA) was as high as 900 pmol/mg on birth, but decreased to about 200 pmol/mg on day 7 after birth. At the same time, protein and nucleic acid oxidation products also showed a peak on birth and significantly decreased on day 7. These results further confirm that large amounts of free radicals are produced

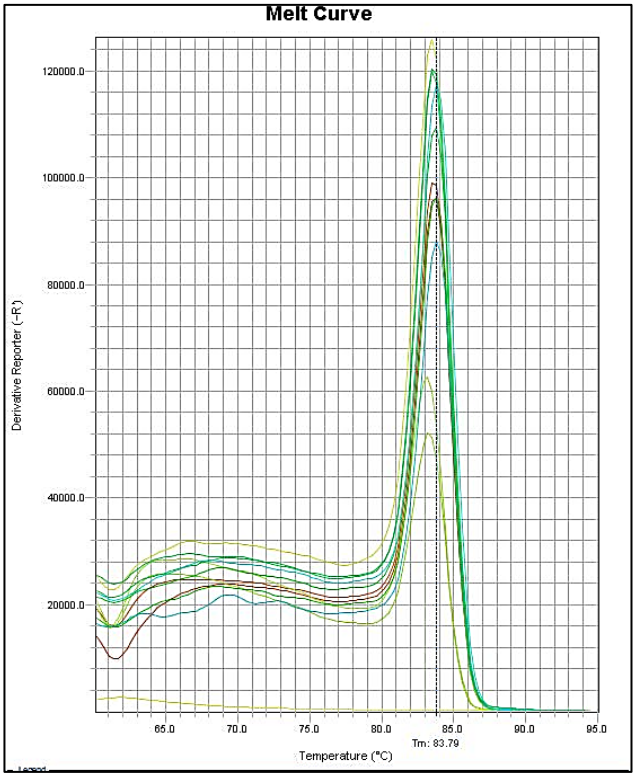

Fig 6 Melt curve plot NRF2 gene

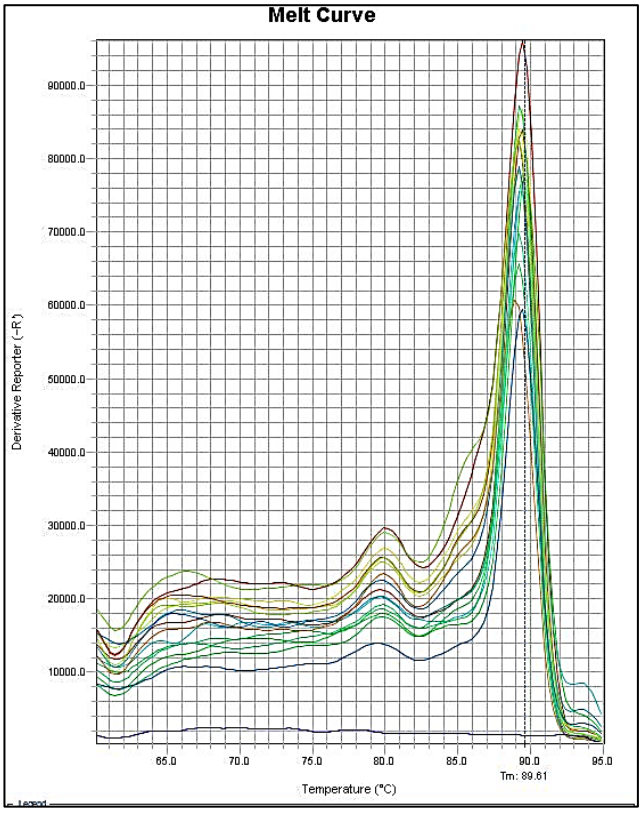

Fig 7 Lipid peroxidation MDA level nMol /mg of Hb in indigenous adult pigs and piglets

during the birth process of piglets, and the weak antioxidant system cannot timely clean up the free radicals induced by birth stress, thus causing the oxidative stress response of piglets (Yin et al., 2013). It was further reported by Luo et al., (2016) that the content of oxygen free radical hydrogen peroxide (H_2O_2) significantly increased after weaning, while the activities of antioxidant enzymes such as GSH-Px and SOD were significantly inhibited.

This is reflected in present study in which NRF2 gene expression was found higher in the adult indigenous group who have lower values of thyroxin levels in established reference intervals than piglets.

Metabolic adaptation responses are essential to preserve energy homeostasis for basic functions in the organism during fasting and reflect metabolic capacity of the animals. During fasting, bioenergetics intermediate sensors (NAD+, AMP, CoA) initiate cellular stress-sensing pathways that further activate oxidative stress protective responses mediated through mainly NRF2. Activation of the NRF2 pathway occurs under the influence of a wide variety of intrinsic or extrinsic stresses that include by-products of metabolic processes (e.g., ROS), exposure to chemical/environmental agents and conditions of nutrient disturbances such as fasting, overfeeding etc. (Tebay et al., 2015). This adaptive capacity mediated through NRF2 protective pathway is well regulated in adult animals compared to young animals same has been reflected in the present study in which higher NRF2 gene expression was found in the adult pigs compared to piglets.

Conclusion

Thus, present findings suggest that NRF2 gene expression levels are effective molecular markers for determining antioxidative capacity in indigenous pigs and need to be investigated as molecular markers about modulating and improving the antioxidant capacity in indigenous piglets and adult pigs.

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Disclaimers/Conflict of interest

The authors hereby declare that there is no conflict of interest regarding the experimental data and manuscript.

Informed consent

Necessary ethical permission was obtained from Institutional Animal Ethical Committee (IAEC) vide no. IAEC/19/21/KNPCVS/2022 dated 05/07/2022 before conducting the actual research trial.

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