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Study on association of sirtuin 1 and 3 gene expression with metabolic profile of indigenous pigs and piglet

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Abstract
Poor metabolic capacity affects overall health status of piglets and it is mainly associated with various piglet associated health problems. Several studies have cited the role of histone deacetylating enzymes called sirtuins, mainly sirtuin 1 and 3 in regulating nutrient sensing pathways, caloric restriction response and thus regulating overall cellular metabolism of animals. The research was conducted to study the expression level of sirtuin 1 and 3 genes in blood samples in indigenous piglets (N=8) relative to adult pigs (N=8) using de novo custom synthesized primers of sus scrofa species. Beside this serum metabolic profile was tested and compared between piglets and adult indigenous pigs. The data of the gene expression study were correlated with serum metabolic profile to assess the significant correlation of metabolic health status of pigs with sirtuin gene expression. The expression of sirtuin1 gene was found significantly lower ($p< 0.01$) and expression of sirtuin 3 gene was found significantly higher ($p< 0.01$) in piglets relative to adult indigenous pigs. Significantly lower values ($p< 0.01$) of total protein, albumin, globulin, calcium and significantly higher values ($p< 0.01$) of triglycerides, alkaline phosphatase, phosphorus, lactate dehydrogenases were found in indigenous piglets as compared to adult pigs. The values of total protein, albumin was found positively correlated ($p> 0.05$) and triglycerides, phosphorus and alkaline phosphatase ($p> 0.05$) were found negatively correlated with sirtuin I fold expression level. Values of glucose ($p> 0.05$), BUN, triglyceride, ALP and lactate dehydrogenase ($p> 0.01$) were found positively correlated and values of calcium and albumin ($p> 0.05$) were found negatively correlated with sirtuin 3 fold expression level. It is concluded that the sirtuin 1 and 3 expression levels varies in two age groups of indigenous pigs and are also associated with overall metabolic status of pigs.

Keywords: Sirtuin, metabolic, indigenous pigs

Introduction
Indigenous pig production contribute major portion of total pig production in India. The pig production system suffers from economic losses in terms high piglet mortality rate, low birth weight of piglets, poor metabolic capacity of piglets despite of these constrains indigenous pig production is popular as these sector provide marginal profitability due to less input cost and some inherent advantages of pig production i.e. high fecundity rate, excellent feed conversion efficiency, early maturity, and short generation interval. Besides incidences of sow crushing, respiratory issues, hypoglycemia, and infections, piglets also experience a range of stressors, including intrauterine nutrient deficiency, weaning stress, and farrowing stress, which can lead to poor metabolic capacity, growth rate, immunity, and nutrition, as well as higher incidences of mortality. Poor metabolic capacity in piglets is a major contributing factor to all of these health conditions, and early measures in pig rearing are necessary to determine and modulate piglet metabolic stress and reduce losses in pig production.

Metabolic status of the animals is mainly dependant on age, sex, breed, nutrition, stress, health, disease and physiological state of animal. At cellular level metabolic capacity is regulated by class of nutrient sensing molecules called as Sirtuins. Sirtuins are nicotinamide adenine dinucleotide-consuming histone deacetylating enzymes involved in various biological processes (Lin *et al.*, 2018) [12]. Sirtuin 1 and 3 are physiological modulator of metabolism. Defects in the pathways controlled by SIRT1 and SIRT3 are known to result in various metabolic disorders. Consequently, activation of sirtuins by genetic or pharmacological means can elicit multiple metabolic benefits that protect mice from diet-induced obesity, type 2 diabetes, and nonalcoholic fatty liver disease. (Nogueiras, R *et al*. 2012) [15]. Based on occurrence within the cell, there are seven subtypes of Sirtuin (SIRT1 to SIRT7). Out of this SIRT3, SIRT4, and SIRT5 are found in mitochondria, SIRT1, SIRT6, and SIRT7 are found in

the nucleus while SIRT2 is found in cytoplasm (Hsu *et al.*, 2018) [7]. SIRT1 and SIRT3 are the two most notable sirtuins engaged in mammalian energy homeostasis and various metabolic pathways. SIRT1 along with SIRT3 regulates glucose homeostasis, insulin secretion, and mitochondrial biogenesis (Pacifici *et al.*, 2019) [16]. Sirtuin 3 has been found to be associated with regulation of oxidative stress and antioxidant response. SIRT1 were found to protects against a decline in vascular endothelial function, metabolic syndrome, ischemia-reperfusion (IR) injury, obesity and cardiomyopathy, and SIRT3 is protective against dyslipidemia and IR injury. (Kane A. & Sinclair D. 2018) [9]. Considering the importance of sirtuin1 and 3 in the regulation of metabolic pathways cited in various animals research, the present research was conducted to study the possible role of Sirtuin 1 and 3 in regulating the overall metabolic status in pigs as well as to study the relation of age related variation in sirtuin gene expression and metabolic status in piglets and adult pigs.

Materials and Methods

The experiment was conducted on total 16 apparently healthy indigenous pigs (8 Piglets of below 6 months of age and 8 adult pigs above two years age) maintained at college farm. The pigs were restrained in a lateral recumbent position and approximately 6 ml blood was collected from medial femoral artery with the help of sterile needle and syringe. Whole blood and serum samples were collected for real time PCR gene expression and serum biochemical profile study respectively.

Biochemical Study: Serum biochemical parameters of pigs *viz* total glucose, total protein, Albumin, Globulin, Creatinine, BUN, SGOT, SGPT, Ttoal bilirubin, Direct Bilirubin, Cholesterol, triglycerides, ALP, GGT, LDH, Calcium, Phosphorus, uric acid were studied using standard biochemical kits of TrueChemie India Pvt. Ltd. on Semi Auto Biochemistry Analyzer (ALTA Chem 220 USA).

Sirtuin gene expression study-

Relative gene expression study of sirtuin 1 and 3 gene in indigenous piglets and adult pigs was carried out by real time PCR was carried out using SYBR green based method.

Total RNA extraction and quality determination

Total RNA was isolated using trizol reagent (SRL). Trizol reagent and chloroform were added to PBMCs pellet and mixed gently followed by centrifugation at 12,000 rpm for 15 min at 4 °C. The initial upper aqueous layer was aliquoted into sterile microcentifuge tubes and equal volume of ice cold isopropanol was added, vortexed gently followed by centrifugation at 12,000 rpm for 12 min. 4 °C. Pellet was washed twice with 75% ethanol by centrifugation at 7500 rpm for 5 min at 4 °C. The isolated total RNA was stored in nuclease free water at 4 °C (Qiagen, India). Purity of RNA was checked by using Nano-Drop spectrophotometer (Thermo). 2 µl of dissolved RNA was added to find out the ratios of 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

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

Primers- De novo primers were synthesised using genescript real time PCR primer design software tool against Sus scrofa Sirtuin 1 and 3 gene from the available sequence in the NCBI data base. Sus scrofa B actin was used as endogenous control in the study. The sequences and expected polymerase chain reaction (PCR) product lengths are shown in Table 1. Specificity of designed primer were confirmed by NCBI BLAST analysis.

List of oligonucleotide primers for Real time PCR

Sr. No.	Gene	F/R	Sequence (3′-5′)	Product size (bp)
1	Sirtuin 1	Forward	ACTGTGAAGCTGTACGAGGA	140
		Reverse	GGCTCTATGAAACTGCTCTGG	
2	Sirtuin3	Forward	ACCGCGCCTCTTGGTTTA	143
		Reverse	ATTGGGTCACAAAGGCCG	
3	β Actin	Forward	CGGGACCTGACCGACTACCT	185
		Reverse	CGGGCAGCTCGTAGCTCTTC	

Quantitative Real-time PCR (qPCR) was performed with Invitrogen Sybr green ®Supermix kit. The qPCR conditions were as follows, initial denaturation at 95 ºC for 30 s, annealing at 58 ºC for 10 s and lastly extension at 72 ºC for 15 s for 35 cycles.

The cyclic condition use for Real time PCR

Segment	Remark	Thermal Profile	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	

No template control (NTC) was placed for gene quantification for checking the contamination in the reaction components other than the cDNA. After the run has ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the “dissociation curve” method of the real time machine (Applied Biosystem, USA). The specificity of real time PCR products were checked by analysis of melting temperature (Tm) of the product obtained from dissociation or melting curve and by 1.5% agarose gel electrophoresis to verify the exact amplicon size. Mean ΔΔCt of sirtuin 1and 3 gene expression of indigenous piglets relative to the Adult indigenous pigs was compared.

All values which are obtained were expressed as mean ± SEM (standard error of the mean). Statistical analysis of the experimental data was carried out according to the method described by Snedecor and Cochran (1994) [5]. Significant differences in the level of serum metabolic parameters were tested by independent sample t test. Critical alpha level of < 0.05 was used for statistical significance using SPSS statistical tool software version 22. Correlation between sirtuin1 and 3 gene expression in blood samples and various serum biochemical parameters were determined by Karl pearsons coefficient of correlation. A Significant correlation was mentioned at 0.05 and 0.01 significance levels.

Results and discussion

Serum metabolic profile

Values of mean ± SE of various serum metabolic parameters of piglets and adult pigs studied has been represented in the table 3. Levels of total protein, albumin, globulin, and calcium values were significantly lower in indigenous piglets than in adult indigenous pigs, whereas triglycerides, alkaline phosphatase, phosphorus, and lactate dehydrogenases values were significantly higher. Values of all the serum parameters are within reference range provided for indigenous pigs of India by Kumar *et al*. (2017) [8] and Mili B *et al*. (2020) [14]. Statistically non-significant higher serum glucose levels were found in piglets. Piglets may have greater glucose levels due to their increased basal metabolic rate, which corresponds to higher amounts of thyroxin and adrenocortical hormones observed during their growth phases. Weaning stress causes greater cortisol levels in piglets, which may also lead to higher glucose levels. Total proteins and albumins were significantly lower in the piglets than adult indigenous pigs. These results are in agreement with earlier reported results by Yu *et al*. (2019) [22]. Tothova *et al*. (2021) [21] studied protein profile during post natal period in piglets. He reported marked developmental alterations in the serum protein pattern in piglets along with the age. Animal protein profiles are altered by genetic, dietary, and hormonal variables. Lower plasma protein levels directly reflect the liver's biosynthetic capability and protein consumption, whereas greater plasma protein levels, especially albumin, suggest beneficial anabolic effects. Plasma protein levels rise with age, peaking in healthy adult animals. Lower protein levels in pigs may be associated with thyroxin's catabolic action on serum proteins. Growing animals often have increased ALP activity. In the current study, ALP levels were shown to be greater in piglets than in adult pigs, indicating that bone metabolism is higher throughout the growth period. The enzyme serum alkaline phosphatase is thought to be a measure of bone tissue turnover. Piglets were shown to have greater plasma triglyceride levels than adult pigs in the current study. Higher plasma triglyceride levels may be attributable to higher amounts of lipolytic hormones and lower adipose tissue mass in developing pigs. Friendship *et al*., 1984 [4] had similarly reported that values of serum protein increased with the age. Also, Klem *et al*., (2010) [11] who studied biochemical parameters in Norwegian grower pigs and compared them with piglets also reported same trend in the piglets compared to adult pigs. Lactate dehydrogenase is regarded as a measure of muscular activity, and its levels rise as muscle metabolism rises. Higher LDH levels in pigs correspond to greater muscle turnover during the growth period, which is influenced by different anabolic and catabolic hormones that regulate muscle metabolism and, ultimately, muscle mass. In this study we found calcium levels were non-significant among piglets and adult pigs but higher phosphorus levels were found in the piglets compared to adult pigs. Other serum biochemical profiles *viz*. BUN Creatinine, SGOT, SGPT, Cholesterol, GGT and uric acid did not differ significantly between the two age groups of indigenous pigs.

Table 1: Serum Metabolic profile of adult indigenous pigs and Piglets

Sr. No.	Parameter	Adult Pigs	Piglet	P value
1	Glucose (mg/dl)	101.85 ± 4.75	113.56 ± 6.75	0.178
2	Total Protein (g/dl)	7.25 ± 0.19	5.84 ± 0.25**	0.001
3	ALBUMIN (g/dl)	4.09 ± 0.18	2.96 ± 0.25**	0.003
4	GLOBULIN (g/dl)	3.16 ± 0.26	2.88 ± 0.16	0.386
5	BUN (mg/dl)	10.60 ± 0.62	12.39 ± 0.65	0.068
6	CREATININE	0.92 ± 0.08	0.83 ± 0.05	0.372
7	SGOT (U/L)	43.56 ± 4.52	59.76± 7.52	0.086
8	SGPT (U/L)	52.47 ± 3.13	55.24 ± 3.75	0.580
9	Total Bilirubin (mg/dl)	0.43 ± 0.05	0.54 ± 0.050	0.154
10	Direct Bilirubin (mg/dl)	0.29 ± 0.05	0.35 ± 0.04	0.321
11	Cholesterol (mg/dl)	106.22 ± 3.23	114.88 ± 4.17	0.123
12	Triglyceride (mg/dl)	29.78 ± 1.51	39.07 ± 3.54*	0.030
13	ALP (U/L)	178.31 ± 11.27	321.13 ± 35.39**	0.002
14	Ca (mg/dl)	11.03 ± 0.27	9.39 ± 0.27**	0.001
15	P (mg/dl)	5.65 ± 0.29	7.29 ± 0.28**	0.001
16	LDH (U/L)	479.11 ± 25.62	675.84 ± 38.04**	0.001
17	GGT (U/L)	45.50 ± 3.29	50.21± 3.46	0.341
18	Uric Acid (mg/dl)	0.39 ± 0.06	0.43 ± 0.03	0.548

Gene expression study of sirtuin 1 and 3 gene in indigenous pigs and piglets

The target gens sirtuin 1 and 3 and Endogenous control gene β-actin PCR product amplification were confirmed by agarose gel electrophoresis. Agarose gel electrophoresis of PCR product (Fig no.1) was used to verify the bp size of the specific amplified PCR product using 50 bp DNA ladder. Melt curve analysis conducted after real-time PCR amplification run revealed a single melt peak in all tested target genes along with an amplification plot (fig.no.2 & fig. no.3).

Gene expression fold (2^-ΔΔct) and log 2 fold relative mRNA expression in Adult indigenous pigs relative to piglets of target genes *viz* sirtuin 1 and sirtuin 3 have been presented in Table 4 & 5, respectively. Also, Gene expression fold (2^-ΔΔct) and log 2 fold relative mRNA expression in Adult

indigenous pigs relative to piglets of target genes *viz*. sirtuin 1 and sirtuin 3 has been graphically represented in fig. no. 4, respectively.

Table 2: Sirtuin 1 fold gene expression and log fold gene expression change in Adult indigenous pigs relative to piglets (Mean ±SE)

Sr. No	Groups	Fold expression	Log fold gene expression change	Gene expression fold difference ratio	P value
1	Adult	1.225 ± 0.22	5.65 ± 1.03	4.88	0.01
2	Piglet	0.247 ± 0.052	1.147 ± 0.237		

In this study we found mRNA expression of sirtuin 1 gene was significantly lower ($p< 0.01$) in indigenous piglets relative to adult indigenous pigs. Sirtuin 1 is a cytosolic deacetylating protein and it is considered as important metabolic regulator enzyme. Sirtuin 1 enzymes have been linked to the beneficial effects of calorie restriction. Physiological adaptation to starvation requires higher activity of SIRT1 as well as suppression of thyroid hormone (TH) action to achieve energy conservation (Cordeiro *et al.*, 2013) [3]. Higher thyroid hormone levels found in young piglets compared to adult pigs may be reflected in lower sirtuin 1 activity compared to adult indigenous pigs.

The expression of the sirtuin 3 gene was found to be considerably higher ($p< 0.01$) in indigenous piglets than in adult indigenous pigs in the current study. Sirtuin 3 is a mitochondrial enzyme that has a function in mitochondrial biogenesis and dynamics (Torrens-Mas *et al.*, 2019) [20]. Enhanced sirtuin 3 activity leads to enhanced oxidative phosphorylation in mitochondria, whereas increased mitochondrial activity creates reactive oxygen species, which leads to increased lipid peroxidation of cellular membranes. Thus, increased mitochondrial oxidative phosphorylation may be one cause of the pigs' greater oxidative stress. Sirtuin 3 has been shown to defend against oxidative stress and aid in the removal of ROS from the cell (Kawamura *et al.*, 2010) [10]. SIRT3 has been substantiated to be involved in almost all aspects of mitochondrial metabolism and homeostasis, protecting mitochondria from a variety of damage (Zhang *et al*. 2020) [23].

Table 5: Sirtuin 3 fold gene expression and log fold gene expression change in Adult indigenous pigs relative to piglets (Mean ±SE)

Sr. No	Groups	Fold expression	Log fold gene expression change	Gene expression fold difference ratio	P value
1	Adult	0.0463 ± 0.018	0.0438± 0.017	0.039	0.03
2	Piglet	1.21 ±0.505	1.105 ± 0.164		

Correlation sirtuin 1 and 3 gene expression study in indigenous piglet and pigs with serum metabolic profile

A correlation study of fold expression of sirtuin 1 and sirtuin 3 genes in indigenous piglets relative to adult indigenous pigs with various tested values of metabolic parameters revealed a significantly high correlation with target genes. Values of total protein and albumin were found positively correlated ($p> 0.05$ significance level) with fold expression level of sirtuin 1 gene and Triglycerides, alkaline phosphatase was found negatively correlated with sirtuin 1-fold expression level. Also, values of phosphorus were found negatively correlated ($p> 0.01$ significance level) with fold expression level of sirtuin 1 gene.

In present study Values of glucose was found positively correlated ($p> 0.05$ significance level) with fold expression level of sirtuin 3 gene and values of calcium and albumin were found negatively correlated with sirtuin 3-fold expression level. Also, values of BUN, triglyceride, ALP and Lactate dehydrogenase were found positively correlated ($p> 0.01$ significance level) with fold expression level of sirtuin 3 gene.

There was a negative connection between serum glucose and sirtuin 1 levels. Sirtuin 1 controls blood glucose levels by inhibiting GLUT4 transcription and increasing insulin secretion (Haigis *et al*., 2010) [6]. SIRT1 overexpression in mice is associated with favourable metabolic outcomes, such as lower adiposity and serum cholesterol, as well as enhanced resistance to obesity-induced glucose intolerance and insulin resistance (Qiang *et al*., 2010 & Bordone *et al*., 2006) [17, 1]. The current investigation discovered a non-significant negative correlation between sirtuin 1 gene expression and blood cholesterol level. ALP enzyme levels exhibit a substantial negative correlation with sirtuin 1 expression, which might explain why piglets have lower sirtuin 1 expression and greater ALP enzyme levels. ALP levels rise with bone demineralization, catabolism, and accelerated bone tissue turnover, which happens in bone diseases such as rickets and osteomalecia. Thus, the current findings show that sirtuin 1 expression is important for modulating ALP enzyme levels.

There was positive correlation has been observed between sirtuin 1 expression and serum calcium level in present study. Lu *et al.*, (2020) [13] reported that vitamin D supplementation upregulated sirtuin 1 level. Thus, there is positive correlation between sirtuin 1 expression and serum Vit D3 and calcium levels. There was significant negative correlation ($p> 0.01$) observed between sirtuin 1 expression and serum phosphorus level in present study. It was reported that sirtuin 1 retards hyperphosphatemia induced vascular calcification thus sirtuin 1 has protective effect against adverse effects of hyperphosphatemia (Lu *et al*., 2020) [13]. There was a significant positive connection ($p> 0.01$) between serum BUN levels and sirtuin 3 expression. Glutamate dehydrogenase and ornithine transcarbamylase is a crucial metabolic enzyme essential for urea production that is found in the mitochondrial matrix with SIRT3. *In vitro* and *in vivo*, SIRT3 deacetylates and activates GDH (Schlicker *et al*., 2008) [18]. Overall, sirtuin 3 activation stimulates amino acid and protein catabolism, resulting in reduced total protein and albumin levels in the blood. As a result, there was a substantial negative connection between sirtuin 3 expression and serum total protein and albumin levels. A substantial negative connection ($p> 0.05$) was detected between serum calcium and sirtuin 3 levels. It might be attributed to increased calcium absorption by tissues. The levels of lactate dehydrogenase enzyme and sirtuin 3 had a strong negative connection ($p> 0.01$). Glycolysis inhibition has been seen during sirtuin 3 activation, which may contribute to reduced LDH levels with sirtuin 3 activation. Sirtuin 3 deficiency were found to exacerbates diabetic cardiomyopathy by increasing

LDH level and decreasing ATP level in cardiac myocardium of diabetic mice (Song *et al* 2021) [19]. SIRT3 deacetylates one or more proteins of the electron transport chain complex I, including NDUFA9, according to a recent research. Complex I activity is decreased in SIRT3 knockout mice and increased in SIRT3 overexpressed mitochondria. (Cimen *et al* 2010) [2].

Table 4: Correlation of Sirtuin 1 and 3 fold gene expression with serum metabolic Profile

Sr. No	Groups	Fold expression sirtuin 1	Significance value	Fold expression sirtuin 3	Significance value
1.	Glucose	-0.299	0.26	0.506*	0.045
2.	Total Protein	0.577*	0.019	-0.456*	0.076
3.	Albumin	0.525*	0.037	-0.499*	0.049
4.	Globulin	0.181	0.501	-0.03	0.912
5.	BUN	-0.346	0.189	0.624**	0.010
6.	Creatinine	-0.055	0.838	-0.165	0.542
7.	SGOT	-0.212	0.430	0.378	0.149
8.	SGPT	-0.056	0.837	0.053	0.845
9.	Total bilirubin	-0.262	0.327	0.47	0.066
10.	Direct bilirubin	-0.005	0.986	0.261	0.329
11.	Cholesterol	-0.224	0.405	0.047	0.863
12.	Triglyceride	-0.465*	-0.465	0.497*	0.050
13.	ALP	-0.501*	0.048	0.452*	0.079
14.	Ca	0.520*	0.039	-0.577*	0.019
15.	P	-0.785**	0.000	0.426	0.100
16.	LDH	-0.433	0.094	-0.630**	0.009
17.	GGT	-0.065	0.812	0.314	0.236
18.	Uric acid	-0.338	0.201	0.217	0.419

*. Correlation is significant at the 0.05 level (2-tailed)

**. Correlation is significant at the 0.01 level (2-tailed)

Conclusion

The contribution of the piggery sector to overall meat production in India is low, and there is a need to improve the sector through various input measures. Overall, the study highlights the need to address metabolic stress in piglets to improve the sustainability and profitability of pig production in India. Serum metabolic profile reflects overall metabolic status of the animals. Study shown that there is significant differences in the levels of some of the serum biochemical parameters between indigenous piglets and pigs reflecting variation in metabolic status or capacity in these two age groups. At gene expression level significantly lower expression of sirtuin1 gene and significantly higher expression of SIRT 3 gene were found in piglets compared to adult indigenous pigs. These sirtuin expression were found to be highly correlated with serum metabolic profile parameters. Thus expression of the histone deacetylating sirtuin 1 and 3 may be playing important role in regulating the overall metabolic status in pigs.

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