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Identification and Association of Genomic Variants of *TNP1* and *TNP2* Genes with Sperm Motility in *Bos indicus* Breeding Bulls

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KHA collected samples and performed the experiments and wrote the initial draft. AN designed and supervised the experiment. MJ, AR, and AN analyzed the data and finalized the manuscript. All the authors have contributed in reading and critical discussion of the manuscript.

Key words
Infertility, *TNP1, TNP2*, Sperm motility, Bulls

ABSTRACT

During spermiogenesis, round spermatids are transformed into motile spermatozoa by undergoing multiple processes. One of these processes in differentiation process of spermatozoa is chromosomal condensation in which histones are first replaced by transition proteins1 and 2 which later are replaced by protamine proteins. The compact packaging of sperm chromatin is essential for maintaining sperm DNA integrity, sperm motility, capacitation, acrosome reaction, normal embryogenesis and birth of healthy offspring. In present study, total 4 SNPs in *TNP1* gene and 9 SNPs in *TNP2* gene were identified. All of four SNPs were identified in upstream region in *TNP1* gene while out of 9 SNPs in *TNP2* gene, one downstream variant, six SNPs in exonic regions including 2 synonymous at position rs9927982 and rs9927976, 4 missense variants at position rs9927957, rs9927816 and rs9927797 were identified. Splice region mutation at position rs9926701 and 3' UTR variant at position rs9926542 was also identified. Association between these identified SNPs and semen motility was evaluated. One SNP at position rs109072682 in *TNP1* gene was significantly associated with sperm motility while out of 9 variants of *TNP2* gene, two SNPs, at position 9926697 and at position 9927816 were also found associated with sperm motility while in TNP gene expression analysis, TNP1 was identified with the highest expression *TNP1* (FPKM value:1477.86) followed by *TNP2* (1053.4), *TNPO1* (36.88), *TNPO2* (27.06), *TNPO3* (22.57), and *ETNPPL* (0.04). It is the first report that revealed the differential expression of *TNP* gene family and statistical association of upstream and exonic SNPs with sperm motility in *TNP* genes in Sahiwal breeding bulls.

INTRODUCTION

In livestock industry, fertility traits are of great economic value as they impart vital impact on production (Abdollahi-Arpanahi *et al*., 2017), beef producer's profitability and determines the maintenance of livestock farms (Harris, 1970). Reproduction being highly complex trait involves multiple events like gametogenesis (gametes development), fertilization, uterine attachment, embryogenesis, and fetal development (Abdollahi-Arpanahi *et al*., 2017). A remarkable percentage of reproductive failure is associated with subfertility of bull because of poor quality of semen and/or health status (DeJarnette *et al*., 2004) hence evaluation and selection for only female fertility traits have huge consequences.

Gametogenesis in bulls (spermatogenesis) has been divided into three phases: spermatocytogenesis, meiosis and spermiogenesis. Spermatocytogenesis involves, consistent cycles of mitotic cell divisions in germ cells of seminiferous tubules to renew stem cells, produce spermatogonia, primary spermatocytes and increasing the yield of spermatogenesis. Meiosis results in duplication, reshuffling of genetic material, two cell divisions that reduce the chromosome number to half and yield four haploid round spermatids. During spermiogenesis the round spermatids are differentiated into fully mature flagellated spermatozoa that are released into the lumen of seminiferous tubules (Staub and Johnson, 2018).

Spermiogenesis can be divided into two phases in vertebrates as well as in many other animals. During the first phase of spermiogenesis, there is round nucleus, histones are present as major basic nuclear proteins and

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is transcriptionally active. In the second phase dramatic changes in chromatin structure, nuclear shaping, and condensation of chromatin take place. After nuclear elongation starts, histones are removed and are replaced by another set of proteins called protamines which are now acting as the major nuclear protein and transcription ceases in many species of animals (Oliva and Dixon, 1991). In mammals, another set of proteins named transition nuclear proteins (*TNPs*) act to be intermediate proteins in this histone to protamine replacement process. The spermatozoa during fertilization not only deliver the male genome to oocyte but also perform many other functions related to embryonic development. During spermiogenesis, the spermatozoa develop a highly organized genome. The sperm DNA is compacted about 85% by protamines and 15% by histones which carries epigenetic signals, different kind of RNA molecules, numerous proteins that participate in post-fertilization events especially in development of embryo. About 50% of infertility cases are referred to as idiopathic infertility (Amor, 2019).

Spermatogenesis involves replacement of histones by protamines in many species (Ausio, 1999). In fishes and birds, this replacement of histones by protamines occurs directly (Oliva and Dixon, 1991) but in mammals and some marine vertebrates this replacement involves intermediate proteins named transition nuclear proteins (Wouters-Tyrou *et al*., 1998) Transition proteins are present in many mammals including rat, mouse, boar, ram, cattle and human (Akama *et al*., 1996; Chevaillier *et al*., 1998; Steger *et al*., 1998). Only two major transition nuclear proteins, *TNP1* and *TNP2*, are eminent in spermatids of rodent and other mammals (Grimes *et al*., 1977). During the compaction of sperm DNA, histones are first replaced by transition nuclear proteins 1 (*TNP1*) and transition nuclear proteins2 (*TNP2*) that are subsequently replaced by protamine 1 (*PRM1*) and a precursor of protamine 2 (*PRM2*), which is then processed to its mature form by proteolysis (Chauviere *et al*., 1992). This chromatin remodeling is accompanied by the conversion of negatively supercoiled nucleosomal DNA into a non-supercoiled state and changes in nuclear shape (Ward *et al*., 1989), introduction of transient DNA breaks (McPherson and Longo, 1993) and chromatin condensation.

TNP1 is an 6200 Da protein having about 20% arginine and 20% lysine that are uniformly distributed and no cysteine (Kistler *et al*., 1975). TNP2 has 13,000 Da molecular weight along with about 10% arginine, 10% lysine, and 5% cysteine (Grimes *et al*., 1977). It has a highly basic C-terminal domain and an N-terminal domain that forms zinc fingers (Meetei *et al*., 2000). TNP1 is abundantly expressed protein in spermatozoa (Heidaran *et al*., 1988) and has highly conserved sequence in various mammals (Kremling *et al*., 1989) while the sequence of TNP2 is poorly conserved (Alfonso and Kistler, 1993) and its expression levels and protein abundance varies among different species (Steger *et al*., 1998). Both proteins (TNP1 and TNP2) are encoded by *Tnpl* and *Tnp2* single-copy genes, respectively. The *Tnp2* gene is closely linked to the two protamine (*Prm*1 and *Prm*2) genes (Engel *et al*., 1992) that suggesting their arousal by gene duplication and also might have retained common functions. In contrast, *Tnpl* is located on a separate chromosome (Heidaran *et al*., 1988).

TNPs are exclusively localized to nuclei of elongating and condensing spermatids (Meistrich, 1989). They are first detected in step 10–11 spermatids (Alfonso and Kistler, 1993; Heidaran *et al*., 1988). They reach maximal levels during steps 12–13 during constituting 90% of the chromatin basic protein with TNP1 level being 2.5 times higher than those of TNP2 (Yu *et al*., 2000) and are not detected in the nucleus of spermatid after the early part of step 15 (Alfonso and Kistler, 1993; Heidaran *et al*., 1988). Since the TNPs are constituting 90% of the basic chromatin proteins in condensing spermatids (Yu *et al*., 2000), they should be highly important. Different functions have been attributed to the TNPs like histone removal, nuclear shaping, transcriptional repression, chromatin condensation and repair of the DNA strand breaks that transiently occur during the nucleosomes removal (Caron *et al*., 2001). TNP1 Firstly destabilize nucleosomes and prevent DNA bending, both of which contribute to displacement of histones (Baskaran and Rao, 1990; Levesque *et al*., 1998). Secondly, the zinc fingers of TNP2 particularly bind to CpG sites and could be responsible for the global repression of RNA synthesis (Kundu and Rao, 1996). Thirdly, both TNPs are supposed to act as alignment factors for DNA strands and TNP1 is involved in the DNA strand breaks repairing (Boissonneault, 2002; Caron *et al*., 2001). Fourthly both TNPs cause condensation of DNA but TNP2 is more effective (Baskaran and Rao, 1990; Brewer *et al*., 2002; Levesque *et al*., 1998). Abnormal TNP2 protein results in acrosomal defects and inability of the spermatozoa to penetrate the zona pellucida causing male infertility (Yu *et al*., 2000). Inactivation of TNP2 in mice causes the failure in transduction of the PRM2 and a sub-fertile phenotype that exhibits less condensed DNA in sperm nuclei and increased level of DNA breaks (Yu *et al*., 2000). The objective of the study was to investigate the genetic variability of the *TNP1* and *TNP2* genes in Sahiwal breeding bulls and to evaluate the expression and association between these identified SNPs with semen motility.

MATERIALS AND METHODS

Semen straw samples (n= 100) of Sahiwal breeding

bulls were collected from semen production unit (SPU), Qadirabad, Sahiwal with their recorded phenotypic data of sperm motility. These semen straw samples were transported in liquid nitrogen and stored in the freezer at −20 ºC before DNA extraction. For separation of sperm cells from seminal plasma, semen samples were centrifuged at 15,000 rpm for five min as a result sperm cells were settled at bottom in the form of pallet while semen plasma as supernatant. For separation of traces of sperm and cellular debris, the seminal plasma was centrifuged and the supernatant was separated from sperm pallet. Phenol-chloroform method (Sambrook *et al*., 1989) was employed to extract genomic DNA with slight modifications using mercaptoethanol and proteinase K and the isolated DNA was dissolved in TE buffer. The purity, quality and concentration of genomic DNA isolated from semen samples were checked by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA samples having the OD_{260}/OD_{280} ratio of 1.83±0.005 were used for further work. The isolated DNA was stored at a temperature of −20 ºC. Primers for exonic regions of *TNP1* and *TNP2* genes were designed using PRIMER3 software (https://primer3.org/) from the sequence accession number NC_032651.1 and NC_037352.1, respectively, available on NCBI. The primers specificity was confirmed using in-silico PCR. Amplification of the genes was done with the help of synthesized forward and reverse primers using polymerase chain reaction (PCR). The total volume of the polymerase chain reaction was 25 μL, which included 2 μL template DNA (100 ng/μL), 2.5 μL 10x PCR buffer with 2.5 μL $MgCl_2$, 1 μL of each forward and reverse primer (both at 10 mol/L), 2.5 μL of dNTPs, 0.5 μL of 5U of Taq DNA polymerase, and 13μL of nuclease-free water (Thermo Fisher Scientific). The PCR amplification was performed as follows: Initial denaturation step at 94ºC for 3 min, followed by 35 cycles of denaturation step at 94ºC for 30 sec, annealing step at 59ºC for primer set for 30 sec and extension at 72ºC for 1 min and final extension step at 72ºC for 10 min. The PCR products were stored at −20ºC refrigeration. The quality and size of amplified PCR products were confirmed by using 1.2 percent agarose gel electrophoresis by using gel documentation system. PCR amplified products were purified using ethanol precipitation while sequencing by the ABI Genetic analyzer 3130 XL (Applied Biosystems, USA). Alignment of sequences was performed by BLAST and CHROMAS bioinformatics tools with reference gene sequences *Bos indicus* (NCBI reference sequence: NC_032651.1) and *Bos taurus* (NCBI reference sequence: NC_037352). Using 4Peaks software (https://nucleobytes.com/) background noise in the amplified PCR product sequence chromatogram was trimmed from both sides and pairwise alignment was obtained with NCBI ref. seq. accession numbers NC_032651.1 and NC_037352.1 using NCBI online BLAST software. MEGA6 (http://www.megasoftware.net) was utilized to analyze SNPs in the *TNP1* and *TNP2* genes amplified from breeding bulls.

TNP1 *and* TNP2 *gene expression analysis*

Testis samples of Sahiwal cattle bulls (n=3) were collected from slaughterhouse located in Lahore cantonment, Pakistan. Sahiwal bulls were 25 and 27 months old. The testis sample of each bull was obtained by veterinary doctor. After removing the epididymis, fat and fascia tissues, three slices of testicular samples were cross-cut from the middle of testis and immediately immersed into liquid nitrogen and stored until total RNA extraction. Total RNA for each sample was isolate by using TRIzol reagent. The concentration, purity and integrity were assessed to meet the requirement of the Illumina HiSeqTM 2500 sequencing. The first step of the research was to use HISAT2 to match raw RNA-seq data to the ARS-UCD1.3 bovine genome assembly. Data integrity was ensured by implementing quality control methods. Following alignment, StringTie was used for transcript assembly and quantification, which made it easier to get expression levels for specific genes. In the downstream study, relevant statistical approaches were incorporated into the Ballgown R program to examine differential gene expression. R was used to create heatmaps and volcano plots as well as to visualize expression patterns and perform pertinent statistical analysis. To investigate the expression of TNP gene family, FPKM data were extracted and shown using ggplot2 in a bar plot.

RESULTS AND DISCUSSION

The *TNP* gene family in cattle have different expression patterns, according to the transcriptome research. *TNP1* (FPKM value: 1477.86) was the most highly expressed member of the *TNP* gene family. It was followed by *TNP2* (1053.4), *TNPO1* (36.88), *TNPO2* (27.06), *TNPO3* (22.57), and *ETNPPL* (0.04). The comparison within gene family reveals the member's different levels of expression, suggesting possible functional differences. Interestingly, *TNP1* was substantially more expressed than the other members of the *TNP* family. These findings underscore the importance of exploring gene family dynamics in understanding cattle transcriptomic responses.

During spermiogenesis, chromatin remodeling and chromosomal condensation is essential for normal sperm morphology, sperm motility and of course fertilization. Chromosomal condensation is the function of sperm nuclear proteins including transition proteins

and protamines. So genomic mutations in these genes, could result in abnormal chromosomal condensation and infertility. In present study, in total, 4 SNPs in *TNP1* gene and 9 SNPs in *TNP2* gene (Table I) were identified. One out of four mutations C>T (p.2682) in *TNP1* gene was statistically significantly associated with sperm motility. Pardede *et al*. (2024) reported positive correlation in *TNP1* and *TNP2* genes protein abundance with motility, velocity, mitochondrial membrane potential (MMP) and fertility in bulls. Three SNPs (G>A, C>T, C>A at 205, 340 and 346 bp, respectively) in the intronic region of *TNP1* gene were observed between variant-C and variant-D of Murrah buffalo that significantly affected the maturation of spermatozoa and infertility. The significantly higher percentage of immature spermatozoa were observed in variant-D than in variant-C (Panigrahi and Yadav, 2010). A 15 nucleotides deletion, encompassing the recognition site for CRE transcription factor, was identified in the 5'-promoter region of the TNP1 gene in infertile men causing reduced expression of TNP1 gene and human male infertility (Miyagawa *et al*., 2005). Heidari *et al*. (2014), identified a novel nucleotide substitution (g.IVS1+75T>C) in intronic region of *TNP1* gene in 15 infertile men while this mutation was absent from the normozoospermic men. Hirenallur *et al*. (2019) observed a deletion of 'G' nucleotide in 3′UTR region of *TNP1* showing non-significant effect on sperm motility in both crossbred and purebred cattle. Two SNPs g.528G>A (rs1388116558) and g.442A>G (rs110469441) were identified in 3'UTR of *TNP1* gene in Chinese Holstein bulls. These mutations were present in bta-miR532 and bta-miR-204 binding sites, respectively. The g.442 A>G and g.528 G>A altered the binding of bta-miR-204 and bta-miR-532 to the 3'-UTR of TNP1 and resulted in translational suppression influencing the morphological characteristics sperms of Chinese Holstein bulls (Zhang *et al*., 2015). A significantly lower expression levels of TNP1, TNP2 and HILS1 transcripts in spermatozoa were identified in asthenozoospermic men as compared to normozoospermic men (Jedrzejczak *et al*., 2007).

Out of 9 SNPs in *TNP2* gene, one downstream variant: p.5298 was identified at position 9925298. The upstream and downstream variants can alter transcription factors binding sites that of course can alter regulation of TNP2 protein expression. In an investigation Gao *et al*. 2014, demonstrated a g.C1536T SNP located in 39-UTR, the microRNA (miRNA) binding site of the *TNP2* gene altered the expression of miRNAs and their targets. Bioinformatics study suggested location of g.C1536T SNP in 39-UTR of *TNP2* gene in the bta-miR-154 binding region. The genotypes CT and CC showed significantly higher expression of TNP2 mRNA as compared to TT genotype with respect to g.C1536T site. A significant association between g.C1536T polymorphism located in TNP2 39-UTR (altered the binding of TNP2 with bta-miR-154) and semen quality traits was found in Chinese Holstein bulls.

Table I. List of identified SNPs in *TNP1* and *TNP2* gene and their association analysis with sperm mortality in Sahiwal cattle.

Gene	SNP ID	SNP location	Genotype (Mean±SE)			P value
			AA	AB	BB	
TNP1	p.2682C>T	Intronic	55±3.01	58±3.03	69±1.74	0.0044
	p.2589T>C	Intronic	52±1.89	51±1.51	58±2.22	0.0707
	p.2567G>A	Intronic	43±1.06	43±1.03	46±1.02	0.0976
	p.2409T>C	Intronic	40±2.57	39±2.34	43±2.56	0.6578
TNP2	p.5298	Downstream	39±2.58	39±1.68	44±1.23	0.1732
	p.6542 C>T	3' UTR	35±1.64	38±1.84	37±2.15	0.5158
	p.G128A	Exonic	45±4.25	39±2.34	54±1.83	0.0160
	p.6701T>C	Splice Region	39±2.70	38±1.84	37±2.15	0.8369
	p.S66I	Exonic	38±2.27	41±1.58	40±1.83	0.5712
	p.T60A	Exonic	42±4.10	36±1.68	54±1.83	0.0019
	p.A13P	Exonic	34±1.40	38±2.17	35±2.45	0.4339
	p.Q6Q	Exonic	38±2.52	41±2.71	40±2.69	0.7742
	p.K4K	Exonic	42±1.85	45±1.78	40±2.69	0.6369

In present study, identified 3' UTR variant p.6542 is highly important because 3' UTRs of mRNAs are involved in the regulation of messenger RNA based processes like localization of mRNA, stability of mRNA and its translation. It can also mediate the 3'UTR dependent protein-protein interactions and controls the protein complex formation, post-translational modifications and alterations in protein conformations. To investigate the involvement of 3'UTR of TNP2 in its translational regulation, a transgenic mice (Tnp2-hGH transgene) was developed in which 3'UTR of TNP2 was replaced by 3'UTR of human growth hormone. In this mice simultaneous transcription and translation was observed in spermatid providing the evidence of involvement of 3'UTR of TNP2 in repression of its translation. So this premature mRNA translation resulted in sperm head abnormality, reduction in sperm motility and infertility in male mice. The stage specific and strictly time related mRNA translation of *TNP2* gene is essentially required for proper spermatid differentiation in spermatozoa (Tseden *et al*., 2007).

Six SNPs in exonic regions, including 2 synonymous p.K4K (Lys to Lys) at position rs9927982, p.Q6Q (Gln to Gln) at position rs9927976, 4 missense variants p.A13P at position rs9927957 and amino acid change Ala to Pro, p.T60A at position rs9927816 and amino acid change Thr to Ala, p.S66I at position rs9927797 and amino acid change Ser to Ile, p.G128A at position rs9926697 and amino acid change Gly to Ala while splice region mutations p.6701 at position rs9926701 was identified. Two SNPs p.G128A at position 9926697 and p.T60A at position 9927816 were found associated with sperm motility trait (Table I). These missense mutations could result in conformational alterations in TNP2 protein and resulting in loss of function phenotype of TNP2 protein resulting in defects in chromosomal condensation during spermiogenesis and other secondary defects due to abnormal TNP2 protein while splice region mutations could disrupt RNA splicing causing the loss of exon or the inclusion of introns and changing the coding sequence for proteins. Three SNPs g.G269A in exon1, g.C480T in intron1 and g.C1536T in 39-UTR in the *TNP2* gene were found in 392 Chinese Holstein bulls and significant association between semen quality traits and these three polymorphisms was identified. The higher sperm motility with haplotype combinations H6H4, H6H6, and H6H8 was observed than H7H8 and H8H4 haplotype combination (Gao *et al*., 2014). To identify already reported SNPs analysis of SNPs g. 480 C>T and g. 1536 C>T, a PCR-RFLP analysis was performed in Sahiwal, Tharparkar and Karan Fries cattle bulls by Ranjan *et al*. (2018), and identified only C480T SNP in the samples population of these bulls. Two SNPs at 182 bp and 186 bp position in intronic region of *TNP2* gene through PCR-SSCP technique were identified in Murrah buffalo bulls. A nonsignificant association between the impact of these two SNPs and individual motility, mass activity and maturation of sperms was found while animal's breed demonstrated the significant impact on individual motility and mass motility but nonsignificant on maturation of sperms (Panigrahi and Yadav, 2009). A 237C>T polymorphism in intronic region of *TNP2* gene showed significant association with initial progressive motility in purebred while with post thaw motility in overall cattle bull population in study (Hirenallur *et al*., 2019). Nikitkina reported a SNP g.1536 C>T in TNP2 gene having non-significant association with semen quality traits in Russian Holstein bulls. A significant correlation of GG genotype at rs199536093 in *TNP2* with idiopathic azoospermia in comparison with normal homozygote was identified and revealed protective impact on fertility while other SNPs C>G genotypes at position rs199536093 and C>T at position rs11640138 had nonsignificant association with infertility in men and *TNP2* rs199536093 C>G demonstrated over dominance effect (Ghadirkhomi *et al*., 2022). Two missense mutations c.301C>T and c.391C>T in TNP2 gene were reported by Imken *et al*. (2009) in infertile patients but no significant association with infertility trait. Similarly, Heidari *et al*. (2019), also identified c.301C>T and c.391C>T polymorphism in patients having varicocele infertility but again no significant difference of these SNPs was identified between patients and fertile individual. But mutation g.IVS1 G-26C at position rs8043625 in intron, expressed significant difference between varicocele infertile and fertile individuals. Miyagawa *et al*. (2005), identified five missense polymorphisms 129C>T, 188G>C 246G>A, and 357A>C and 518C>T in a case-control study of infertile and fertile individuals resulted in amino acid change R28C, G47H, G67S, K104Q and R131W, respectively. But no significant difference between fertile and infertile individuals was recognized. Similarly, Siasi *et al*. (2012), also found nonsignificant association between G1272C SNP in TNP2 gene and azoospermia or oligozoospermia.

A *TNP2* null mice model experiment was performed to investigate the importance of *TNP2* in spermatogenesis and chromosomal condensation process. The abnormalities observed were the slight increase of sperm retention in stage IX to XI tubules, increased tail morphology in epididymal sperms and slight DNA breaks. But in *TNP2* null mice spermatogenesis process was normal (Zhao *et al*., 2001). In another study, Zhao *et al*. (2004), demonstrated the impact of loss of both TNPs i-e, *TNP1* and *TNP2*, on spermatogenesis and chromosomal condensation in mice. In TNPs null mice showed DNA breaks, irregular chromosomal condensation and loss of post transcriptional

Table II. Differential expression of *TNP* gene family.

S. No.	Gene ID	Gene symbol	FPKM Sahiwal case	TPM Sahiwal case	Read count Sahiwal case	Norm read count Sahiwal case
1	767620	TNPO1	36.88	63.53	1639	1262.531
2	281538	TNP2	1053.4	1814.46	4504	3469.4543
3	515186	ETNPPL	0.04	0.06	1	0.7704
4	504589	TNPO2	27.06	46.6	1942	1495.9325
5	281537	TNP1	1477.86	2545.59	4397	3387.0316
6	537183	TNPO3	22.57	38.87	1055	812.6719

processing of protamine and high number of sperm retention in testis, reduction in epididymal sperms, increased abnormal sperms and sterile spermatozoa were observed. Another *TNP2* null mice study performed by Adham *et al*. (2001), supports the results described by Zhao *et al*. (2001). In this study, Adham *et al*. (2001) observed total infertility in *TNP2* null mice on the inbred 129/Sv background while completely normal fertility on the mixed background C57BL/6J*120/6J. Shirley *et al*. (2004), demonstrated abnormal sperm chromatin condensation, reduced motility, abnormal sperm morphology, DNA degradation and reduced protamine processing and reduced expression of TNP proteins in epididymal spermatozoa in nine possible genotypes of *TNP1* and *TNP2* null mice. Fewer sperm abnormalities were observed in double heterozygous males as compared to homozygous for a single TNP null mice.

The differential expression of *TNP* gene family was identified in transcriptome analysis in Sahiwal cattle bulls in present study. *TNP1* (FPKM value: 1477.86) was found to be the most highly expressed member of the *TNP* gene family followed by *TNP2* (1053.4), *TNPO1* (36.88), *TNPO2* (27.06), *TNPO3* (22.57), and *ETNPPL* (0.04) (Table II). These findings underscore the importance of exploring gene family dynamics in understanding cattle transcriptomic responses. Rosa-Xavier *et al*. (2021), observed higher expression of *TNP2*, *PRM1* and *17β-HSD3* genes in an analysis of cell differentiation histopathological study on bovine testicular parenchyma which supports the conviction of direct involvement of these genes in differential development of germ cells during bovine spermatogenesis. Transition proteins 1 and 2 showed positive correlation with sperm quality traits (motility, velocity, MMP, and fertility) (Pardede *et al*., 2024). In a transcriptomic profiling of testicular tissue, Elango *et al*. (2020), identified higher expression of *TNP1* and *TNP2* genes in crossbred bulls. The differential expression of both TNP1 and 2 was observed by Omolaoye *et al*. (2022), in all disease groups (non-obstructive azoospermia, obstructive azoospermia, non-obstructive and obstructive azoospermia, spermatogenic dysfunction, sperm dysfunction, and Y chromosome microdeletion). A significantly higher expression of *TNP2* gene was observed in successful spermatozoa retrieval group of azoospermic patients and a significant association was also identified between TNP2, PRM2, JMJD1A and successful spermatozoa retrieval (Amjad *et al*., 2021). A significantly decreased expression of TNP1 gene was observed in chronic stress group of rats affecting the pathological changes in spermatogenic cells (Tian *et al*., 2021). In a quantitative analysis, Jedrzejczak *et al*. (2007), identified reduced expression levels of *TNP1, TNP2* and *HILS1* genes mRNAs in asthenozoospermic patients.

CONCLUSION

TNP1 and *TNP2* genes are well known for their involvement in semen quality and fertility parameters, including chromosomal condensation and maturation. However, studies are yet to examine the *TNP1* and *TNP2* gene in indigenous Sahiwal cattle breed in Pakistan. Therefore, present study was conducted to identify the genomic variants of *TNP1* and *TNP2* in indigenous Sahiwal cattle breed bulls. We identified one SNP in *TNP1* gene and two SNPs in *TNP2* gene associated with sperm motility. In expression analysis, *TNP1* and *TNP2* genes among other TNP gene family member showed higher expression. Our research identified valueable findings, but small sample size impeded in the way of comprehensive analysis. So we propose more extensive studies with the larger sample size for precise confirmation of the obtained results in breeding bulls.

DECLARATIONS

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IRB approval

Non-invasive sampling procedures were conducted;

therefore, ethical approval was not required.

Ethical statement

This study has been conducted in full compliance with ethical standards. The research followed all relevant guidelines to ensure the welfare of the animals. As non-invasive sampling procedures were used, so no ethical approval was required for this research work.

Data availability

The data will be openly available to all readers.

Statement of conflict of interest

The authors have declared no conflicts of interest.

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