Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.083

RESEARCH ARTICLE

Comparative Transcriptomic Analysis of Spermatozoa from Xiangxi and Simmental Bulls under Heat Stress: Implications for Fertility Prediction

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ARTICLE HISTORY (22-279)

Received: August 12, 2022
Revised: October 24, 2022
Accepted: October 27, 2022
Published online: November 27, 2022

Key words:
Semen
Fertility
Transcriptome
Xiangxi cattle
Simmental cattle

A B S T R A C T

In the current study, a total of six 5 year old healthy bulls reared in heat stress zone were used and semen from these bulls was collected by artificial vagina and undergoes for semen quality analysis. Results of semen quality parameters showed that the average semen volume and sperm density of Simmental bulls were 9.3mL and 1.23×10^9mL, and sperm acrosome integrity rate were lower as compared to Xiangxi cattle bulls. The transcriptome analysis revealed a 36.8Gb clean data and the obtained clean data of each sample was reached 5.84Gb. The transcriptome analysis further revealed that the percentage of q30 base was 91.19% or above. Based on the comparison results, 4,890 new genes were found, including 2,451 functional annotations. Go analysis explored that GO annotation system included 3 main sections that were biological processes, molecular functions and cellular components, respectively. In conclusion, results showed that the average semen volume and sperm density of Simmental bulls was higher and sperm motility and sperm acrosome integrity rate was lower as compared to Xiangxi cattle bulls. This study has developed a transcriptomic profile of spermatozoa of Xiangxi cattle and Simmental cattle bulls and reported Xiangxi cattle and Simmental cattle bull's spermatozoa change the gene expression involved in various biological process. This study could provide the experimental basis of poor fertility in Xiangxi cattle bulls and provide possible fertility biomarkers.

To Cite This Article: Li J, Wang X, Sun A, Li H, Luo Y, He F, Huan C, Zhou X, Li C, Zhang B, Yi K, 2023. Comparative transcriptomic analysis of spermatozoa from Xiangxi and Simmental bulls under heat stress: implications for fertility prediction. Pak Vet J, 43(1): 184-188. http://dx.doi.org/10.29261/pakvetj/2022.083

INTRODUCTION

Domestic livestock breeds are providing basic resources like meat and milk to increasing population of humans. In China, Xiangxi cattle, is a native breed of southwestern China, reared for beef production and is well known due to its better meat quality, good immunity and ability to resist against hot and humid climate.

Although, Xiangxi cattle is known for heat resistance capacity, but it has been reported that cattle fertility is considered to be adversely affected in hot arid climates (Gwazdauskas, 1985; Brito *et al.*, 2002; Prastowo *et al.*, 2019). Previously, it has also been reported that not only extremes climatic conditions like temperature, humidity and radiation but wind also influences negatively on reproduction (Gwazdauskas, 1985). Current study only focused on sperm traits and transcriptome sequencing in two bull's species under heat stress and we mainly focused on heat stress influence on sperm traits. Heat stress in animals is defined as the condition at which body mechanisms activated to maintain animals body thermal balance after exposing to intolerable higher temperature (Marai and Haeeb, 2010). Previous study explored that elevated temperature not only alter reproductive functions but also influence spermatogenesis and reduced fertility at different extents (Kim *et al.*, 2013). Similarly, another study reported that fertility is reduced during heat stress as a result of dysfunctions in reproductive processes (De Rensis and Scaramuzzi, 2003) and even a slight higher temperature negatively influences on semen production. Heat stress also have harmful effect on spermatogenesis and also decreases testosterone levels (Murray, 1997).

Even relatively small variations or fluctuation in environmental temperature or conditions may alters bull's fertility, which is of a great concern for cattle production in developing countries where animals are mostly reared in open environment. Keeping in view the global warming, the current increase in temperature may have great implications for cattle reproduction and production in the near future even in countries with a temperate climate (Rojas-Downing *et al.*, 2017).

Therefore, for accurate diagnosis of bull's fertility and cattle reproduction, understanding and use of modern techniques that can access semen quality are required (M. K *et al.*, 2019; Saraf *et al.*, 2020; Douglas *et al.*, 2021). Keeping in view this background, the current experiment was carried out to develop spermatozoa transcriptomic profile of Xiangxi and Simmental cattle's and to distinguish the sperm quality and transcriptomic variations between Xiangxi and Simmental bulls. It was hypothesized that sperm quality difference will be existed between Xiangxi and Simmental bulls, and transcriptomic profiling of spermatozoa collected from Xiangxi and Simmental, would support to develop tools for fertility prediction of Xiangxi and Simmental bulls.

MATERIALS AND METHODS

Sample collection: In the current study, a total of six 5-year-old healthy bulls (3 Xiangxi cattle: xxh90, xxh93, xxh94; 3 Simmental: xm55, xm56, xm59) were randomly selected from Hunan Bull Breeding Station approved by the government. All managemental practice were ideal as described in the previous studies (Imran *et al.*, 2021; Riaz *et al.*, 2021). Artificial vagina was used for the collection of semen and stored for further analysis.

Semen quality tests: The amount of semen was directly calculated by the round bottom marked tube. For the determination of semen motility, 10μL of the original semen was dropped on the slide and sperm motility was checked at 400X inverted phase contrast microscope. Sperm apoptosis was analyzed by using flow cytometry. For the determination of semen density: the original semen was diluted with normal saline at the ratio of 1:5-1:10 and the semen density analyzer was used to detect the reading. For sperm acrosome integrity rate determination, the sample was stained with Coomassie brilliant blue solution. Before dying, the staining solution was preheated in a water bath (37 ℃, 10 min), and the smear was placed in the dye solution for 8-10 min. the stained smears were washed with distilled water, dried and sealed.

The acrosome integrity rate (%) = (number of intact acrosome sperm / total number of observed sperm) × 100

For sperm deformity rate smear was formed as described earlier, then he prepared smears were observed under the microscope (400 times oil microscope). At least two smears were made for each sample. More than 200 sperms (divided into left and right areas) were observed in each smear. The average value of the two smears was taken. The coefficient of variation of the two pieces was not greater than 20%. Calculation formula

sperm deformity rate (%) = (number of abormal sperm / overall observed sperms or total number of observed sperm) × 100.

Transcriptome analysis of sperm based on RNA SEQ high throughput sequencing: The sperm of Xiangxi cattle and Simmental cattle bulls were collected, isolated and purified. The mRNA library was constructed and sequenced with Illumina 2,000. The sequencing results were analyzed by database search and differential expression screening, analyzed by DAVID, GO and KEGG pathway databases and screened by gene cards function.

QPCR verification: Primers were designed according to the sequence specificity of the selected differentially expressed genes and synthesized by MDL biotechnology. The relative expression of the gene was calculated by $2^{-\Delta\Delta}$ CT method, and the significance standard was $p < 0.05$.

Statistical analysis: In the current study, Pearson's correlation was used to quantiles/quantile normalization and examination for outliers of experimental data. Transcripts obtained in every single comparison with P value less than 0.01, and false discovery rate less than <20% and folds changes in excess of two were deemed to be significant and further analyses and examined. For the calculation of gene relative expression $2^{-\Delta\Delta}$ CT method was used, and the significance standard was $p < 0.05$.

RESULTS

Semen quality: Due to the difference of bulls breeds, the average semen volume and sperm density of Simmental cattle bulls were 9.3mL and 1.23×10^9mL, which were higher than those of Xiangxi cattle bulls (4.7 and 0.85×10^9ml) (Fig. 1). In terms of sperm motility and sperm acrosome integrity rate, 57% and 60% of Simmental were lower than 77% and 82% of Xiangxi cattle, while the sperm deformity rate of Simmental cattle was 27% higher than that of Xiangxi cattle (Fig. 2). Among Xiangxi cattle bulls and Simental cattle bulls, sperm density and sperm motility did not show statistical differences (P>0.05).

Overview of results obtained after high throughput sequencing: In the current study, transcriptomic analysis of six samples was completed, and 36.81Gb total clean data was achieved. The obtained clean data from every sample was reached to 5.84Gb. The obtained clean data of each sample had %age of q30 base was 91.19% or above. The designated reference genome was used to align the clean reads of every sample and the efficiency of alignment was ranged from 18.62% to 37.11%. On the basis of comparison results, 4,890 new genes were found, including 2,451 functional annotations. Furthermore, gene expression was analyzed which was based on the comparison results. The DEG were found according to genes expression level in each sample, and furthermore, DEG functional annotation and enrichment analysis were also performed.

Repeated correlation assessment: For the biological repetition correlation, Pearson's correlation coefficient R was utilized assessment index. The highest R value equal to 1 shows the higher correlation between the two repeated samples. The correlation statistics of project samples and principal component analysis are shown in Fig. 3.

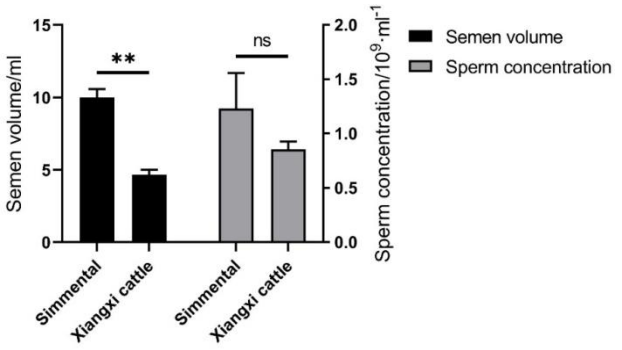

Fig. 1: Sperm volume and concentration of Simmental and Xiangxi cattle.

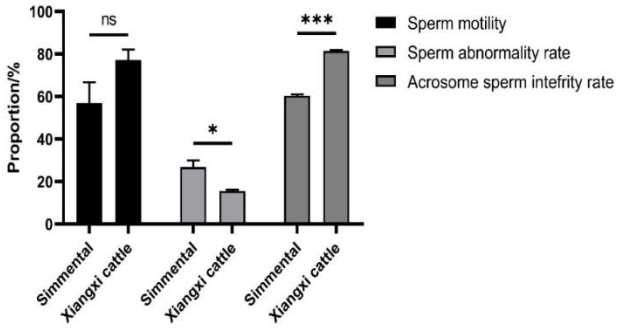

Fig. 2: Sperm motility, sperm abnormality and acrosome sperm interfrity rate of Simmental and Xiangxi cattle.

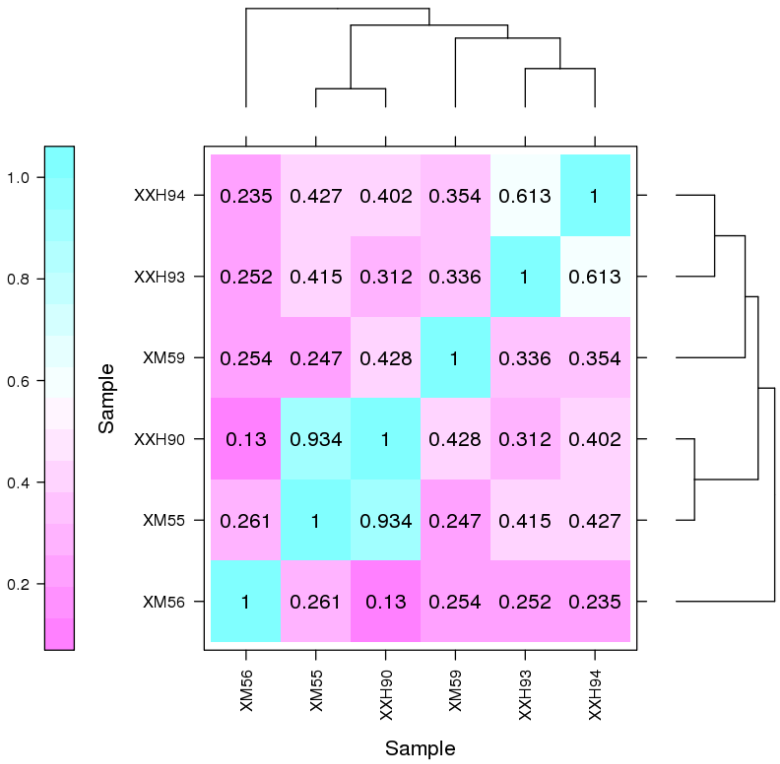

Fig. 3: The correlation statistics of samples and principal component analysis of Simmental and Xiangxi cattle.

GO and KEGG annotation of DEG: It is well known that KEGG is considered as database for gene's function systematic and for genomes information (Chen *et al.*, 2021; Qiu *et al.*, 2018). In the current study, the annotation results of KEGG were classified according to the pathway types in KEGG. The DEGs were mainly distributed in 'cellular processes' and' genetic information processing' and 'metabolism' GO annotation system present acyclic type of graph, that is composed of three main areas that are biological processes, molecular functions and cellular components (Fig. 4). GO annotation system also explored significant proportion difference that also indicates that enrichment tendency of DEG was different from other all genes. These abnormal physiological processes may be one of the reasons for the lack of resistance of Simmental cattle bulls' sperm to heat stress.

DISCUSSION

Increase in temperature along with diet along with diet alteration have impact on cattle physiology, welfare, health, and reproduction (Li *et al.*, 2014; Qiu *et al.*, 2020; Qiu *et al.*, 2021). Very few experiments were carried out to evaluate the effect of heat stress in bulls fertility. Study of correlation of heat stress on fertility is of particularly interest for livestock breeders because it has been recognized as a major cause of sub-fertility (Hansen, 2009). Previous studies has been conducted to evaluate of seasonal effects on bovine semen quality but results of those studies were significantly different from each other (Malama *et al.*, 2017; Sabés-Alsina *et al.*, 2017; Brito *et al.*, 2002).

In the current study, due to the difference of bull's breeds, the average semen volume and sperm density of Simmental cattle bulls were 9.3 and 1.23 × 10^9mL, which were higher than those of Xiangxi cattle bulls (4.7ml and 0.85 × 10^9ml). In terms of sperm motility and sperm acrosome integrity rate, 57% and 60% of Simmental cattle bulls were lower than 77% and 82% of Xiangxi cattle bulls, while the sperm deformity rate of Simmental cattle was 27% higher than that of Xiangxi cattle. These data are also in line with the current situation of cattle breed improvement, because Chinese native yellow cattle have the advantages of good adaptability and

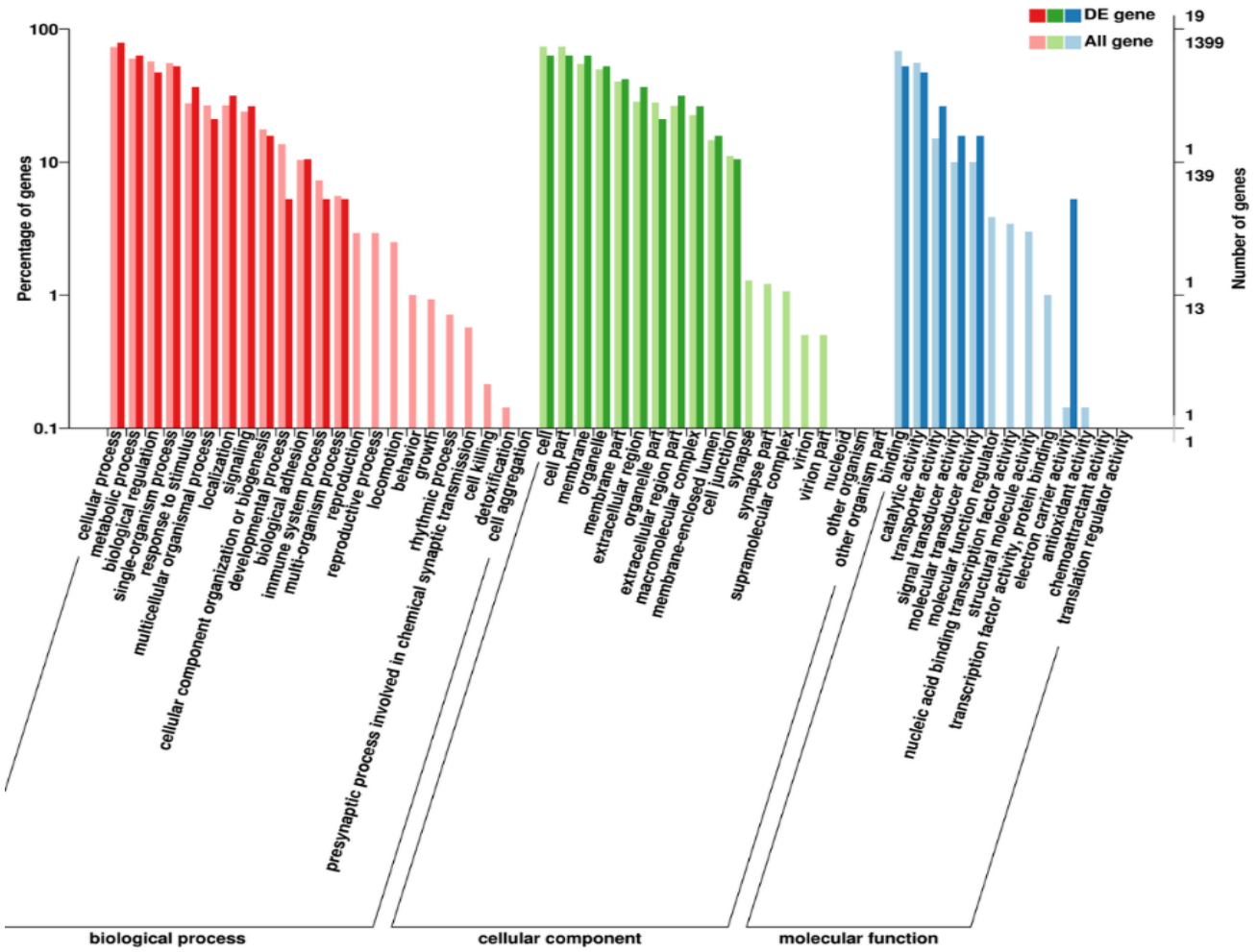

Fig. 4: GO annotation of samples of Simmental and Xiangxi cattle.

strong resistance, but also have the defects of weak production capacity (Lyu *et al.*, 2021). Therefore, the local cattle breeding industry introduces Simmental bulls with better production capacity to crossbreed and improve the offspring of local cattle (Meng *et al.*, 2020).

In the current study, transcript-based alternative technique was used to compare Simmental and Xiangxi bulls' fertility prediction because traditional semen evaluation techniques are uncertain. Previous study has provided the evidence that transcript-based alternative technique is useful to study the function of sperm, spermatogenesis, and embryonic development (Selvaraju *et al.*, 2018). Hence, current study was planned with the objective of comparative transcriptomic profiling of Simmental and Xiangxi bulls spermatozoa to explore the molecular basis for two species bulls fertility and to identify the pathways behind variation between two breeds semen quality and fertility.

The current experiment explored transcripts for 4,890 genes in bulls sperm, as described in the previous studies but the transcript number in our study was much lower than the studies that has been conducted in recent past (Selvaraju *et al.*, 2017; Raval *et al.*, 2019). These variations in transcripts might be due to heat stress on both species because it has already be published that heat stress, sample collection, state pf spermatozoa etc. may influence the transcript numbers (Card *et al.*, 2017).

A recent study has demonstrated that GO and KEGG analysis of all predicated targets of differently expressed gene in fresh and frozen-thawed boar sperm resulted in 438 differentially expressed target genes from transcriptome (Dai *et al.*, 2019) and majority was allocated to the biologicals processes, molecular functions components and cell part and membrane. These findings are correlated with the finding of current study where most of gene were correlated with biological process, molecular function and cellular component (Dai *et al.*, 2019). Prakash *et al.* (2021) further indicated that transcripts that are responsible for oxidative phosphorylation pathway and biological process for example spermatogenesis, and multicellular organism development, are downregulated in crossbred bulls with low fertility. In the current study, transcripts that are responsible for oxidative phosphorylation pathway and biological process were lower in the Xiangxi cattle that were could be reason of low fertility. Similar with Xiangxi cattle, Chinese native yellow cattle have been reported the defects of weak reproduction capacity (Lyu *et al.*, 2021).

It has been reported that sperm transcripts that are upregulated are unique in low-fertile bulls and that are mainly responsible for biological process (Lyu *et al.*, 2021). Similar with the findings of the previous study, KEGG metabolic pathways results of the current study

explored that the sperm transcripts involved in the biological process (translation) were lower in Xiangxi cattle bulls sperm that could be the reason of higher average semen volume and sperm density of Simmental cattle bulls sperm. Similar findings has already been reported in previous study where low fertility bulls were involved in biological process (Card *et al.*, 2017; Selvaraju *et al.*, 2017; Raval *et al.*, 2019) and ribosomal pathway. Findings of the current study, along with the results of previous studies explored the potential variations in the translation machinery of low fertility bulls. Current study along with previous studies has provided the evidence that transcript data may provide the brief description of sperm's functions (Feugang *et al.*, 2010); however, further studies are required to explore the biological process especially translation process in male fertility. The key discovery of current experiment is that sperm transcripts that are involved in biological processes are exceptionally critical, and downregulated in bulls sperm with low fertility.

Conclusions: In conclusion, results showed that the average semen volume and sperm density of Simmental was higher and sperm motility and sperm acrosome integrity rate was lower as compared to Xiangxi cattle. Transcriptomic analysis shows that GO annotation system includes three main branches: biological process, molecular function and cellular component. Furthermore, this study has developed a transcriptomic profile of spermatozoa of Xiangxi cattle and Simmental cattle bull and reported Xiangxi cattle and Simmental cattle bull's spermatozoa change the gene expression involved in various biological process.

Acknowledgements: Authors acknowledge the help of undergraduate students to conduct the current experiment. Authors also acknowledge the help of experimental farms staff for providing assistance. Authors acknowledge the support of Key Research and Development Project of Hunan Province, China (2022NK2025, 2022NK2017) and Innovation Platform and Talent Plan of Hunan Province, China (No. 2019TP 2010).

Authors contribution: JL and XW conceived and designed the experiment. AS, HL,FH and YL carried out the manamgent the cows and bulls. CH and XZ performed invivo experiments and transcriptomic analysis. CL and BZ and KY performed statistcal analysis. All authors reviewed the manuscript.

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