Polish Journal of Veterinary Sciences Vol. 22, No. 2 (2019), 221–225

DOI 10.24425/pjvs.2019.127089

Original article

Missense mutation within cystic fibrosis transmembrane conductance regulator (CFTR) gene is associated with selected parameters of the frozen-thawed sperm in Holstein-Friesian bulls

S. Kaminski[1], D.M. Hering[1], W. Kordan[2], M. Lecewicz[2]

[1] University of Warmia and Mazury, Department of Animal Genetics, Oczapowskiego 5, 10-718 Olsztyn, Poland
[2] University of Warmia and Mazury, Department of Animal Biochemistry and Biotechnology, Oczapowskiego 5, 10-718 Olsztyn, Poland

Abstract

In our previous Genome-wise Association Study we found that Cystic Fibrosis Transmembrane Conductance Regulator gene (CFTR) is a candidate gene for sperm motility in fresh semen of Holstein-Friesian bulls. Since in cows thawed semen is commonly used for the artificial insemination (AI) we have decided to find out whether functional polymorphism within CFTR gene coding sequence is associated with selected parameters of thawed sperm, including their motility evaluated by computer-assisted sperm analysis (CASA), the activity of three antioxidant enzymes: glutathione peroxidase (GPx) catalase (CAT), superoxide dismutase (SOD), ATP content and integrity of sperm membranes. One hundred twenty Holstein Friesian bulls kept in uniform environmental conditions (one AI company) were included in the study. **S**ignificant associations between genotypes of missense mutation within exon 11 of the CFTR gene (Met468Leu) and the activity of antioxidant enzymes and sperm mitochondrial function were revealed. No effect of CFTR genotypes on sperm motility was observed. Significant differences in CAT and SOD activity were found between AA and TT homozygous individuals. Bulls with TT genotype had the lowest activity of both antioxidant enzymes. The same bulls also showed the lowest number of sperm with active mitochondria. Our results demonstrate that missense mutation Met468Leu within CFTR gene is associated with antioxidant enzyme activity and mitochondrial function of bovine thawed sperm without affecting their motility.

Key words: bull, sperm, polymorphism, CFTR gene, antioxidant enzyme activity

Correspondence to: S. Kamiński, e-mail: stanislaw.kaminski@uwm.edu.pl

Introduction

Mutations within Cystic Transmembrane Conductance Regulator gene (CFTR) are commonly known as a cause of one of the most frequent genetic disorder in Caucasian humans – Cystic Fibrosis (CF) (Bear et al. 1992). Men with CF suffer also from obstructive azoospermia (Patrizio and Salameh 1998) as well as non-obstructive azoospermia (Claustres 2005). Jiang et al. (2017) proved that selected mutations within CFTR gene are correlated with non-obstructive azoospermia. CFTR gene is essential for human fertilizing capacity and the impairment of CFTR expression in spermatozoa is associated with lower sperm quality (Li et al. 2010). Influence of mutations in CFTR gene on semen quality was not investigated in livestock species used in artificial insemination, where top sires become fathers of thousands of offspring. In our previous investigation on genome-wide association for low sperm motility (Hering et al. 2014), a significant SNP marker (rs43399120) in chromosome 4 was found, in the close neighbourhood of CFTR. For artificial insemination industry discrete changes in the quality of semen can be a source of economical losses, since they are key factors leading to successful pregnancy (Mathovon et al. 1998). In this paper we hypothesize that polymorphism within CFTR gene might be a genetic marker for semen quality in Holstein bulls.

Materials and Methods

Animals

One hundred twenty Holstein-Friesian bulls from one AI station was included into the study. The bulls were at a similar age (15 months on average) and were kept in uniform feeding and housing conditions. All bulls underwent routine veterinary examinations. No one showed testicle anomalies or any clinical symptoms which could affect semen production.

Phenotypic data collection

Twenty commercial straws of frozen-thawed semen (180 x 10^6 spermatozoa) collected from each bull were used. Straws were produced in weekly intervals. Semen from each sample was centrifuged at 800 x g for 5 min, sperm pellets were separated and washed by re-suspending in 1 ml 0.85% NaCl and re-centrifuging. Further processing of samples to measure the sperm motility by computer-assisted sperm analysis (CASA) and activity of antioxidant enzymes: glutathione peroxidase (GPx) catalase (CAT), superoxide dismutase (SOD) is described in our earlier report (Hering et al. 2015). The results of antioxidant enzymatic activity (U) were calculated for 10^9 sperm cells. Plasma membrane integrity and the mitochondrial energy status were assessed as described by Kamiński et al. (2016). ATP content was measured using a Bioluminescence Assay Kit CLSII (Roche Diagnostics, GmbH, Basel, Switzerland) in accordance with the manufacturer's instructions. The results of ATP content (nmol) were also calculated for 10^8 sperm cells.

Genotypic data and PCR conditions

Genomic DNA was isolated from a half volume of one commercial semen straw using the Wizard Plus Megapreps DNA Purification System (Promega, Madison, WI, USA). A/T missense polymorphism within bovine CFTR gene was chosen (rs208111474), which replaces Methionine by Leucine (Ensembl: ENSBTAG00000006589). A pair of PCR primers (forward 5'TGCAGGCTTCTTGTAGCAGG3'; reverse 5'ACTCAGCACCCCATCTCTGT3') was designed by the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to flank 622 bp fragment of CFTR gene. Genomic DNA from 10 randomly chosen bulls was used to reveal polymorphisms within the amplicon. To identify CFTR polymorphism within a population of 120 bulls, *Hin1*II restriction enzyme was used (Fermentas, Lithuania).

Statistical analysis

A Kruskal-Walis (Statistica v 10.0, StatSoft, Inc.) test was used to check the associations between CFTR genotypes and sperm traits. Multiple comparisons between average ranks for each pair of genotyping groups were calculated. The average of three replicates was calculated for each bull and each semen quality trait. Since bulls were kept in the same environmental conditions (one AI center), were at similar age, and the semen was collected by standardized procedures, the model did not require any corrections for additional fixed or random effects. The relatedness (Identity by Decent) for 120 bulls was 0.0403 (SD=0.058).

Results

Sequencing of exon 11 revealed single nucleotide mutation A/T in position 1402 of CFTR coding sequence (c.1402A>T) (data not shown). This mutation changed the amino acid sequence in position 468 where Methionine was replaced by Leucine (Met468Leu). This amino acid substitution occurs within the protein domain named ABC Transporter-like (Ensembl: ENSBTAG00000006589). The single nucleotide poly-

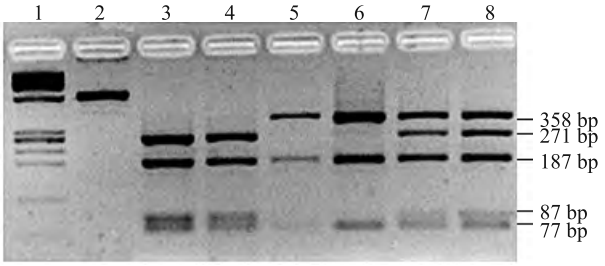

Fig. 1. Genotyping of CFTR A/T missense mutation by Hin1II restriction enzyme. Line 1 - DNA size marker φX174 DNA/HaeIII; Line 2 - product PCR size 622 bp; Line 3,4 - TT genotype; line 5,6 - AA genotype; lines 7,8 - AT genotype; Genotype AA: 358, 187 bp; Genotype TT: 271, 187, 87, 77 bp; Genotype AT: 358, 271, 187, 87, 77 bp.

Table 1. Mean ($\bar{x}$) and standard deviation (SD) for selected semen traits from bulls with particular CFTR genotypes. SOD – superoxide dismutase activity, CAT – catalase activity, GPx – glutathione peroxidase activity, TMOT – total motility, SYBR-14/PI – sperm membrane integrity, JC-1/PI – sperm mitochondrial function, ATP – ATP content.

Sperm trait	All bulls N=120		Bulls with genotypes of c.1402A>T polymorphism						p-value
			AA=18		AT=59		TT=43		
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
TMOT	62.51	6.94	61.21	4.42	63.82	7.24	61.25	7.18	0.075
SOD	5.31	1.04	**5.89a**	**0.95**	**5.28**	**0.99**	**5.10b**	**1.08**	**0.041**
CAT	494.37	138.52	**530.87a**	**114.60**	**514.71**	**142.18**	**451.17b**	**134.43**	**0.017**
GPx	2847.83	420.57	2880.98	378.35	2841.31	440.96	2842.91	417.29	0.305
SYBR-14/PI	72.21	2.72	71.50	2.79	72.29	2.67	72.40	2.78	0.105
JC-1/PI	71.32	1.85	**71.17**	**1.76**	**71.71a**	**1.89**	**70.84b**	**1.74**	**0.045**
ATP	15.59	2.16	15.17	1.26	16.01	2.19	15.17	2.34	0.086

morphism (SNP) is registered in SNPdb (NCBI) under the id number rs208111474. Genotyping of A/T missense mutation in the CFTR gene is illustrated in Fig. 1.

In the population of 120 Holstein-Friesian bulls, three genotypes were identified. AT genotype was the most frequent (49.2%) in comparison to TT (35.8%) and AA (15.0%.). The genotypes of ten randomly chosen samples were confirmed by sequencing (data not shown).

There were no significant differences in CASA sperm total motility between bulls of different CFTR genotypes (Table 1) as well as for remaining CASA kinematic sperm measurements (data not shown).

Significant associations were found between analyzed polymorphism and antioxidant enzyme activities. Sperm produced by bulls with genotype AA had significantly higher CAT and SOD activity than bulls with the TT genotype (Table 1). No significant differences in GPx activity were found. Significant associations were also found between A/T polymorphism and sperm mitochondrial function. The highest number of sperm with active mitochondria occurred in bulls with AT genotype in comparison to TT genotype (p=0.045) (Table 1). ATP content was not associated with CFTR genotype, although the highest value occurred in heterozygote AT, which also showed the highest total sperm motility and the highest number of sperm with active mitochondria.

Discussion

Routine evaluation of the genomic breeding value of dairy bulls generated a huge amount of SNP markers, which has opened the possibility to perform genome-wide associations studies leading to identification of candidate genes involved in traits of interest (Goddard et al. 2016). We used this approach to search for markers involved in genetic variation of specific semen traits and identify significant markers for poor sperm motility in fresh semen and a collection of candidate genes underlying this trait, including CFTR gene (Hering et al. 2014). In the present study, we attempted to check whether the CFTR gene may play any role in frozen-thawed semen of bulls qualified for routine artificial insemination. These bulls produce semen with normal motility (average 62.51%, Table 1) .

To verify the role of CFTR in shaping semen quality traits, a functional polymorphism was found and semen evaluation was undertaken, including sperm motility measurements by CASA, the activity of three antioxidant enzymes, ATP content and the integrity of sperm membrane. The detected Met468Leu polymorphism occurs within ABC transporter domain (within Pfam and Prosite profile) which may potentially affect the overall chloride ion transport. Our analysis showed that total motility and other kinematic sperm parameters

were not affected by CFTR genotypes. It thus appears that freezing and thawing of sperm diminishes the effects of CFTR, especially for semen of standard quality. It should be noted that CFTR was proposed as a candidate gene for poor sperm motility in fresh semen (average sperm motility 27%) (Hering et al. 2014).

The main outcome of our work is that new effects of CFTR polymorphism in thawed semen were discovered. First of all, the activity of two out of three antioxidant enzymes showed significant associations with CFTR genotypes. These associations seem to have an additive mode of inheritance since we observed the most significant differences between alternative homozygotes with heterozygotes showing intermediate values. Although the total number of bulls was not very high, the number of genotypes in each group was sufficient for reliable statistical analysis and possible effect of common ancestor was minimized. It has to be noted that lower activity of SOD and CAT correlated with lower number of sperm with active mitochondria.

Considering the link between CFTR protein and the antioxidant enzymes, we have to look at this protein as a potential source of reactive oxygen species (ROS). Duranton et al. (2012) showed that CFTR is controlling the intracellular ROS out of the cell. Moreover, they demonstrated in a mouse model that the ΔF508 mutation of CFTR led to an impairment of the adaptive erythroid response to oxygen deprivation. This indicates that CFTR is involved in ROS processing. Since ROS production is related to the activity of antioxidant enzymes (Schiff et al. 2006), it can be assumed that CFTR mutation may influence the level of ROS manifested by antioxidant enzyme activity and sperm mitochondrial function. The present results show that in cattle CAT and SOD are more involved in this functional link than glutathione peroxidase. Because the lowest antioxidant activity of the studied enzymes was observed in CFTR homozygote TT, we therefore postulate that sperm from bulls carrying these genotypes are protected against ROS in a less effective way which may affect the mitochondrial membrane. In the present study, we did not demonstrate an association between the GPx activity and CFTR genotypes. It can be explained by species-specific differences in the activity of antioxidant enzymes in the male reproductive system. For example, in stallions, all basic antioxidant enzymes (SOD, CAT, GPx) are present (Baumer et al. 2002). In contrast, in boars, the dominant component of the antioxidant system is a SOD. Furthermore, in these species, low activity of GPx and no activity of CAT were found (Jelezarsky et al. 2008).

Li et al. (2010) revealed that impairment of CFTR expression in human spermatozoa is correlated with a reduction in sperm quality. If we assume that the missense mutation we studied may affect the CFTR protein, it may also decrease the quality of sperm caused by lower antioxidant protection without decreasing sperm motility. Whether this subtle relation affects the final ability of sperm to fertilize the egg should be the subject of future studies.

Conclusion

The missense mutation Met468Leu in CFTR gene is associated with antioxidant enzyme activity and mitochondrial function of bovine sperm without interfering their motility. This is the first report describing association between polymorphism within CFTR gene and bull's semen quality.

Acknowledgements

This work was financially supported by the National Centre of Scientific Research, grant No. N N311 524940 and UWM Olsztyn grant 0105-804. We are thankful to the SHiUZ AI Center (Bydgoszcz, Poland) for access to semen samples and the database of SNP genotypes.

References

Amaral A, Lourenco B, Marques M, Ramalho-Santos J **(2013)** Mitochondria functionality and sperm quality. Reproduction 146: R163-174.

Baumer J, Vo K, Sauber K, Ball BA **(2002)** Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. Theriogenology 57: 1025-1033.

Bear CE, Li CH, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR **(1992)** Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell 68: 809-818.

Claustres M **(2005)** Molecular pathology of the CFTR locus in male infertility. Reprod Biomed Online 10: 14-41.

Duranton C, Rubera I, Cougon M, Melis N, Chargui A, Mograbi B, Tauc M **(2012)** CFTR is involved in the fine tuning of intracellular redox status: physiological implications in cystic fibrosis. Am J Pathol 181: 1367-1377.

Goddard ME, Kemper KE, MacLeod IM, Chamberlain AJ, Hayes BJ **(2016)** Genetics of complex traits: prediction of phenotype, identification of causal polymorphisms and genetic architecture. Proc Biol Sci 283: 1835.

Hering DM, Lecewicz M, Kordan W, Kamiński S **(2015)** Association between ETFA genotype and activity of superoxide dismutase, catalase and glutathione peroxidise in cryopreserved sperm of Holstein-Frieisian bulls. Reprod Domest Anim 50: 168-171.

Hering DM, Oleński K, Kamiński S **(2014)** Genome-wide association study for poor sperm motility in Holstein Friesian bulls. Anim Reprod Sci 146: 89-97.

Jiang L, Jin J, Wang S, Zhang F, Dai Y, Shi L, Zhang S **(2017)** CFTR gene mutations and polymorphism are associated

with non-obstructive azoospermia: from case-control study. Gene 626: 282-289.

Jelezarsky L, Vaisberg C, Chaushev T, Sapundjiev E **(2008)** Localization and characterization of glutathione peroxidase (GPx) in boar accessory sex glands, seminal plasma, and spermatozoa and activity of GPx in boar semen. Theriogenology. 69: 139-45.

Kaminski S, Hering DM, Olenski K, Lecewicz M, Kordan W **(2016)** Genome-wide association study for sperm membrane integrity in frozen-thawed semen of Holstein-Friesian bulls. Anim Reprod Sci 170: 135-140.

Koziorowska-Gilun M, Gilun P, Fraser L, Koziorowski M, Kordan W, Stefanczyk-Krzymowska S **(2013)** Antyoxidant enzyme activity and mRNA expression in reproductive tract of adult male European bison (Bison bonasus, Linnaeus 1758). Reprod Domest Anim 48: 7-14.

Li CY, Jiang LY, Chen WY, Li K, Sheng HQ, Ni Y, Lu JX, Xu WX, Zhang SY, Shi QX **(2010)** CFTR is essential for sperm fertilizing capacity and is correlated with sperm quality in humans. Hum Reprod 25: 317-327.

Mathevon M, Buhr MM, Dekkers JC **(1998)** Environmental, management, and genetic factors affecting semen production in Holstein bulls. J Dairy Sci 81: 3321-3330.

Patrizio P, Salameh WA **(1998)** Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) mRNA in normal and pathological adult human epididymis. J Reprod Fertil Suppl 53: 261-270.

Pena FJ **(2007)** Detecting subtle changes in sperm membranes in veterinary andrology. Asian J Androl 9: 731-737.

Qin C, Yin H, Zhang X, Sun D, Zhang Q, Liu J, Ding X, Zhang Y, Zhang S **(2017)** Genome-wide association study for semen traits of the bulls in Chinese Holstein. Anim Genet 48: 80-84.

Schiff M, Froissart R, Olsen RK, Acquaviva C, Vianey-Saban C **(2006)** Electron transfer flavoprotein deficiency: functional and molecular aspects. Mol Genet Metab 88: 153-158.

Suchocki T, Szyda J **(2015)** Genome-wide association study for semen production traits in Holstein-Friesian bulls. J Dairy Sci 98: 5774-5780.

Thomas CA, Garner DL, DeJarnette JM, Marshall CE **(1998)** Effect of cryopreservation on bovine sperm organelle function and viability as determined by flow cytometry. Biol Reprod 58: 786-793.