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Correlation of Polymorphism of *AQP7* Gene with the Sperm Cells Quality Traits of the Polish Holstein Friesian Bulls

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AK the initiator of the study, who conducted the main experimental part, described the results, wrote the article. IK participated in collecting the research material, who conducted the main experimental part, described the results. ECP performed the statistical calculations, participated in the drafting of the manuscript. CJ, JCJL and AJP result advisors.

Key words
***AQP7* gene, Holstein-Friesian bulls, AQP7 polymorphism, Reproduction, Sperm cells quality, Aquaporins, Aquaglycero-porins, Single nucleotide polymorphisms**

ABSTRACT

The purpose of this study was to evaluate whether single nucleotide substitutions within exon 4 of the quality of AQP7 gene may affect the sperm cells of Polish Holstein-Friesian bulls. The research material was biological material (semen and blood) collected from 60 Holstein-Friesian bulls of the same age. The nucleotide substitutions analyzed are located in exon 4 of the AQP7 gene and are responsible for missense mutations. Individual genotypes were determined using the PCR-RFLP method. Evaluation of sperm quality in terms of concentration, volume, motility, and sperm viability was performed using photometry, flow cytometry, and a computer assisted sperm analyzer. The above studies indicate that all four nucleotide substitutions analyzed in exon 4 of AQP7 gene affect the individual quality characteristics of the analyzed semen of Holstein-Friesian bulls.

INTRODUCTION

Fertility is considered one of the most important economic aspects that determine the profitability of cattle production (Bach, 2018). Despite its fundamental importance, the reproductive efficiency of dairy cattle has dropped dramatically over the past 20 years and is more and more alarming from the point of view of the dairy industry (Turner *et al*., 2021). While female fertility has been given a lot of attention for years (D'Occhio *et al*., 2019; Olsen *et al*., 2020), there are many uncertain factors concerning male fertility, and as research shows (Yin *et al*., 2019), bull's fertility disorders have a significant impact on lowering reproductive efficiency in dairy cattle (Burren *et al*., 2019).

Recent studies has shown that sperm production traits, such as sperm volume and concentration, have moderate heritability (from 0.04 to 0.65 for volume and 0.10 to 0.56 for concentration). In contrast, motility shows an inheritance ranging from 0.01 to 0.51 and 0.07 to 0.35 for sperm abnormalities. The mean heritability values are moderate from volume 0.197, concentration 0.169, sperm abnormalities 0.194 and low for mobility 0.054 (Olsen *et al*., 2020). These results suggest that genetic selection may

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be beneficial.

Aquaporins (AQPs) are integral membrane proteins that play a role in important cellular functions such as cell proliferation, cell differentiation, cell migration, apoptosis and cell adhesion. They act as channel proteins of a plasma membrane and are involved in the transport of water or small molecules such as glycerol and urea (Zannetti *et al*., 2020). In mammals, 13 aquaporin isoforms (AQP0-AQP12) have been identified and found in various organs such as the kidney, lungs, organs of the digestive system, and the skin, as well as in male and female reproductive tissues and gametes, where they play a key role in regulating fluid homeostasis (Fujii *et al*., 2018; Kordowitzki *et al*., 2020).

Within the aquaporin family, so-called aquaglyceroporins were identified. They facilitate the transport of not only of water, but also glycerol, which is widely used as a cryoprotective agent. Therefore, it is assumed that aquaglyceroporins also play a key role in the cryopreservation of mammalian cells. AQP7 is such an aquaglyceroporin (Fujii *et al*., 2018). The aquaporin 7 gene has been mapped to chromosome 8 in cattle (BTA8). This gene consists of 9 exons separated by introns, of which only eight exons can code. The mutations analyzed in the AQP7 gene are located in exon 4, which encodes the second transmembrane region of this protein.

Identifying genomic regions, and most preferably individual genes responsible for bull genetic variability, will improve the understanding of biological pathways for this trait and may indicate opportunities to improve male fertility through selective breeding. Therefore, the aim of this study was to assess whether single nucleotide substitutions within exon 4 of the AQP7 gene may affect the semen quality traits of semen of Polish Holstein-Friesian bulls.

MATERIALS AND METHODS

This study did not require the consent of the local ethics committee. The research material was semen collected from bulls within a production environment at the insemination station and blood collected routinely for animal health monitoring purposes. Therefore, samples taken during routine testing at the breeding station were used. The experiment was carried out for 18 months.

Animals

The research material was 60 bulls of the Polish Holstein-Friesian breed of the same age (±3.5 y.o.). The animals were kept in one station under the same conditions and fed in the same way. All bulls were healthy.

Collection and processing of semen

Semen was collected according to the procedure described by Mizera *et al*. (2019a). Two ejaculates were collected from each bull using an artificial vagina at 7 am. Semen was held in a 37 °C water bath at 37 °C, where the sperm concentration was estimated. The fresh undiluted semen was then microscopically evaluated microscopically (Nikon E 200, China) for mass motility. Subsequently, semen was extended with the commercial BIOXcell® extender without animal protein (IMV Technologies, L'aigle, France) to a final concentration of 140×10^6 spermatozoa/mL, and rated in terms of the percentage and viability of total motile sperm percentage and viability.

Semen was stored in a liquid nitrogen container until analysis and then thawed as described by Mizera *et al*. (2019b).

Evaluation of semen quality

The quality of semen was assessed according to the procedure described by Mizera *et al*. (2019b). A digital photometer (Dr. Lange, LP 300 SDM; Minitube, Tiefenbach b. Landshut, Germany) was used for concentration analysis, sperm motility was assessed using Sperm Class Analyzer (SCA, version 5.1, Microptic, Barcelona, Spain) and a light microscope (Nikon Eclipse E200). A flow cytometer (CytoFlex Beckman Coulter, B3-R1-V0, China) was used to evaluate sperm viability.

Just prior to sperm motility analysis, semen was diluted 1:10 in a warm physiological solution (25 °C) (sodium chlorate 0.9%). Then, 2 µL of the prepared sample was placed in a Leja 4 analysis chamber (Leja Products B.V., Holland) of a thickness of 20.0 µm. The slide was placed on a stage warmer (38 °C).

Double staining of SYBR-14 with propidium iodide was applied (L-7011 LIVE/DEAD Sperm Viability Kit; Invitrogen, Molecular Probes, Barcelona, Spain) using a flow cytometer was applied (CytoFlex Beckman Coulter, B3-R1-V0, China). For this purpose, 50 µL of thawed semen was measured (37 °C for 20 s) and 940 µL NaCl (0.9%) and 5 µL SYBR14 were added. The whole mixture was thoroughly mixed and then incubated (36 °C for 10 min) without light access. Subsequently, 5 µL of PI was reconstituted and incubated 3 min without light, followed by a test.

SNP detection and genotyping

DNA was isolated from the whole peripheral blood (5 ml) collected in vacuum tubes containing K_3EDTA as an anticoagulant using a commercial reagent kit for DNA isolation (MasterPure TM DNA Purification Kit for Blood Version II; Lucigen, Wisconsin, USA) according to the isolation protocol of the manufacturer.

The nucleotide substitutions analyzed are located in exon 4 of the *AQP7* gene and are responsible for

missense mutations (Table I). Individual genotypes were determined using the PCR-RFLP method. To carry out the PCR reaction, a pair of primers designed in Primer3 (http://bioinfo.ut/ee/primer3-0.4.0/) based on DNA sequences from the Ensembl database were used, i.e. forward: 5'- AAAATGGGGAGCTACCGC -3', reverse: 5'- ATCTGTGAAGGGGCAGTC -3'. The underlined G nucleotide in the forward primer was a deliberate change to generate an amplification-created restriction site (ACRS) for the the *Hha*I enzyme for rs477356740 polymorphism.

Table I. SNPs, their locatium, nature of mutation, amino acid change, restrication enzyme (RE), and restriction fragment sizes.

SNPs	Loci	Nature of mutation	AA change	RE	RFLP (bp)
1	*rs477356740* c.205G>C	transversion	Ala69Pro	*Hha*I	G - 348, 18; C - 366
2	*rs458032596* c.233T>C	transition	Val78Ala	*Hin*fI	T - 322, 44; C- 366
3	*rs441112867* c.238A>C	transversion	Ile80Leu	*Bfa*I	A 366; C- 314, 52
4	*rs472401559* c.244G>T	transversion	Val82Leu	*Apa*LI	G - 308, 58; T - 366

AA change, amino acid change; RE, restriction enzyme; RFLP, restriction fragments length polymorphism.

The amplification reactions were carried out in mixtures with a final volume of 25 µl containing: Forward primer (1.0 µl), reverse primer (1.0 µl), standard and ready-to-use 2xPCR Mix (12.5 µl) (A&A Biotechnology, Gdynia, Poland), DNA (2 µl) and nuclease-free water (8.5 µl). Individual PCR steps were carried out under the following thermal conditions: initial denaturation at 94 °C for 5 min, then specific denaturation at 94 °C for 45 seconds, hybridization at 52 °C for 45 seconds, chain extension at 72 °C for 45 seconds (repeated in 30 cycles) and final elongation at 72 °C for 5 min.

The products obtained after PCR (366 bp) were digested with restriction enzymes specific for each of the polymorphisms analyzed polymorphisms (Table I). Then, the obtained fragments were separated using horizontal electrophoresis on 3% agarose gels stained with ethidium bromide and visualized under a UV transilluminator.

Detailed information on the analyzed SNPs, i.e., location, nature of the mutation, amino acid change, restriction enzymes, and fragment size after enzyme digestion, is given in Table I.

Statistical analysis

Statistical analysis of the genetic variants of AQP7 gene and selected semen quality traits of Polish Holstein-Friesian bulls was performed using the STATISTICA®12.0 (2014). A one-way analysis of variance model was used:

$$Yij = \mu + ti + eij$$

Where µ is expected value, ti is analyzed variable and eij, is random error.

For the analysis of the differences among the mean values of the observed characteristics, the Duncan multiple range test was used.

RESULTS

Table II presents the results of the analysis of the relationship between SNP1, SNP2, SNP3, SNP4 polymorphisms and the quality traits of the tested bull semen quality traits. For polymorphisms 1, 3, and 4, no significant differences were observed between animals with different genotypes in terms of volume of the examined ejaculate. Only for the SNP2 polymorphism, homozygous animals with the CC genotype (6.97ml) had the highest semen volume, while the lowest was recorded for animals with the TT genotype (2.63ml) ($P < 0.05$).

In terms of sperm concentration in the ejaculates studied, it was observed that for the SNP1 polymorphism, ejaculates with the highest concentration (2690.71) were obtained from animals with the homozygous GG genotype. These results were significantly different ($P < 0.05$) from the results of other animals with genotypes of TG (1773.15) and TT (906.58). For the SNP2 polymorphism, significant differences ($P < 0.01$) were observed between heterozygous TC and homozygous CC animals, since their sperm concentration varied by 356 million cells/ml of ejaculate on average. For the SNP3 polymorphism, it was also observed that a significantly ($P < 0.01$) lower sperm concentration was obtained from heterozygous AC animals than from homozygous CC animals, and the mean difference was 470 million cells/ml of ejaculate. For the SNP4 polymorphism, the highest sperm concentration was characteristic for animals with the GG genotype (2455.9), while the lowest concentration was recorded in those with the TT genotype (712.24), and these results differed significantly ($P < 0.05$).

The highest percentage of motile sperm in fresh sperm was observed for the SNP3 polymorphism, and was 76.84% for homozygous animals of the CC genotype animals. The lowest sperm motility was observed in samples with TT genotype for SNP4 polymorphism (57.00%). No significant differences ($P < 0.01$) were found between animals tested animals with TT, TC and CC genotypes for SNP2 polymorphism. Furthermore, when analyzing the SNP1, 2 and 4 polymorphisms, sperm motility was at a similar average level. For the SNP1 polymorphism, in the case of frozen semen, animals with

the homozygous TT and GG genotype had significantly lower sperm motility (P <0.01) than animals with the heterozygous TG genotype. When analyzing the SNP2 and SNP4 polymorphisms, no significant differences (P <0.01) were observed in the trait examined trait in animals of particular genotypes. The lowest sperm motility in frozen/thawed semen was observed in bulls with homozygous AA genotype for SNP3 polymorphisms, and it was 44.69%.

Table III presents the results of the analysis of the relationship between the polymorphisms SNP1, SNP2, SNP3, SNP4 polymorphisms and the viability of the sperm viability in frozen/thawed bull semen. When analyzing the SNP1 polymorphism, no significant differences (P <0.01) were observed between the genotypes of the animals and their sperm viability after thawing. For SNP3 and SNP4 polymorphisms, it was observed that animals with the heterozygous GT and AC genotype had the highest percentage of viable sperm and at the same time the lowest non-viable ones. For the SNP3 polymorphism, the differences in mean sperm viability were statistically significant (P <0.01) between animals with the AA and CC genotype, animals with the AC and CC genotype and were 5.98% and 3.82%, respectively. When analyzing the SNP4 polymorphism, the highest percentage of sperm with damaged cytoplasmic membrane was 40.10% in animals with homozygous TT genotype.

DISCUSSION

Tables II and III show the relationship among the SNP1, SNP2, SNP3 and SNP4 polymorphisms and semen quality traits of Polish Holstein-Friesian bulls. Previous studies indicated a relationship between selected polymorphisms and the quality of semen in cattle (Dilbar *et al*., 2019; Wang *et al*., 2020). The genetic selection of animals appears to be of key importance in the context of bovine semen production, which is widely used in AI, which is a dominant reproductive technique.

Until now, numerous studies have been conducted focusing on the analysis of single nucleotide substitutions in the aquaporin 7 gene in relation to bull semen quality. The research focused on SNPs located in exons 2, 3, 4, and 5 (Kumari *et al*., 2016, 2017, 2018) and the authors showed that in cattle the analyzed substitutions were related to acrosome integrity, motility, percentage of viable sperm percentage and post-thaw motility. It has also been shown that the aquaporin 7 gene encoding can be a candidate gene responsible for the quality of freezing of various tissues, including those of the male reproductive system (Kumari *et al*., 2017).

The results of our own research indicate that for the polymorphisms studied, ejaculates with much larger volume and concentration were obtained from animals with a homozygous genotype than from heterozygous animals. Dai *et al*. (2009) obtained similar results for sperm volume and concentration, who showed that bulls with heterozygous BC genotype were characterized by significantly lower semen quality than bulls with BB genotype in terms of examined ejaculate traits. The results of sperm motility before and after freezing presented in Table II showed that the highest percentage of motile sperm was observed in fresh (76.84%) and frozen (51.58%) for the SNP3 polymorphism in homozygous animals with the CC genotype. The results of Liu *et al*. (2011) showed a similar relationship between male heterozygosity (CC, GG, AA) and the percentage of motile sperm in their ejaculates. In the case of AQP7, the analysis of semen of infertile men

Table II. Analysis of the relationship between SNP polymorphisms and tested bull semen quality traits.

SNPs	Genotypes	Volume/ ejaculate(ml)	Sperm concentration (million cells/ml)	Sperm motility (%)	
				Fresh	Frozen
SNP1	TT	3.91±1.20	906.58^{A}±283.92	60.93^{a}±9.77	48.68^{Aa}±4.96
	GG	4.25±1.91	2690.71^{B}±220.76	67.06^{ab}±8.30	47.31^{Aa}±3.88
	TG	4.33±1.99	1773.15^{C}±284.96	68.73^{b}±8.13	50.43^{Bb}±4.19
SNP2	TC	4.74^{A}±0.50	1475.86^{a}±583.21	66.00^{a}±8.45	50.00^{a}±4.51
	TT	2.63^{B}±0.88	1614.94^{ab}±730.20	65.00^{a}±10.97	48.38^{a}±4.56
	CC	6.97^{C}±1.11	1831.63^{b}±621.35	66.58^{a}±7.65	51.36^{a}±3.23
SNP3	AA	4.30±1.67	1289.57^{Aa}±657.35	58.06^{A}±8.71	44.69^{A}±3.40
	AC	4.06±1.82	1704.02^{Ab}±520.86	70.00^{B}±10.41	50.35^{B}±3.99
	CC	4.08±1.77	2174.68^{Ba}±497.61	76.84^{C}±2.48	51.58^{B}±3.36
SNP4	TT	3.90±1.17	712.24^{A}±206.93	57.00^{A}±10.41	46.67^{a}±6.06
	GT	4.17±1.87	1514.32^{B}±272.27	67.38^{B}±7.67	50.11^{a}±4.48
	GG	4.38±1.85	2455.90^{C}±318.57	69.19^{B}±7.76	49.00^{a}±3.82

A, B, C: the least squares means represented by different letters vary significantly (P < 0.01); a, b: the least squares means represented by different letters vary significantly (P < 0.05).

indicated its possible role in maintaining sperm motility (Kasimanickam *et al*., 2017). Other authors (Prieto-Martnez *et al*., 2017a) reported that AQP3 and AQP7 were present in boar spermatozoa (Prieto-Martnez *et al*. 2016, 2017a, 2017b), and that they were involved in sperm cryotolerance (Prieto-Martnez *et al*. 2017b). However, information on AQP in bull sperm remains significantly limited. The results obtained by sperm motility are important from the egg point of view of the fertilization process of the egg, because it is highly dependent on the metabolic activity and motility of sperm cells. Therefore, correct sperm functions are a guarantee of the transport of genetic material transmitted by sperm (Yin *et al*., 2019).

Table III. Analysis of the relationship between SNP polymorphisms and tested bull semen quality traits.

SNPs	Geno-types	Viable sperm %	Sperm of reduced viability %	Non-viable sperm %
SNP1	TT	51.92±10.22	15.94±3.57	32.14±9.71
	GG	50.92±10.59	15.96±3.64	33.12±10.38
	TG	52.42±7.83	16.26±3.36	31.31±7.89
SNP2	TC	53.85±9.92	15.38^{a}±3.30	30.76±9.22
	TT	50.66±7.57	16.34^{ab}±3.15	32.99±8.59
	CC	50.93±8.84	17.72^{b}±4.17	31.34±7.67
SNP3	AA	45.19^{Aa}±8.28	16.32±4.10	38.49^{Aa}±8.88
	AC	54.99^{Ba}±7.88	15.87±3.33	29.13^{Bab}±7.52
	CC	51.17^{ABb}±8.27	16.55±3.10	32.27^{ABb}±8.31
SNP4	TT	43.65^{Aa}±11.89	16.25±3.30	40.10^{Aa}±10.84
	GT	53.72^{Ba}±7.52	16.34±3.59	29.94^{Bab}±7.44
	GG	50.93^{ABb}±9.38	15.71±3.19	33.36^{ABb}±9.25

A, B, C, least squares means represented by different letters vary significantly ($P < 0.01$); a, b: least squares means represented by different letters vary significantly ($P < 0.05$).

The results of sperm viability in frozen semen obtained in our own research showed that animals with the heterozygous GT and AC genotype are characterized by the highest percentage of viable sperm, and at the same time the lowest percentage of nonviable sperm (SNP3 and SNP4). Similar results were reported by Wang *et al*. (2019). However, the identification of factors that affect sperm quality appears to be of significant economic importance for the production of domestic cattle. The low quality of semen as well as the fertility of a given individual has been shown to be associated with the impact of many environmental, physiological and genetic factors (Yin *et al*., 2019).

CONCLUSIONS

The above studies indicate that all four nucleotide substitutions analyzed in exon 4 of AQP7 gene affect the individual quality traits analyzed of the semen of Holstein-Friesian bulls. The analyzed mutations are single nucleotide substitutions, and this type of polymorphism is more and more often a marker. Such SNPs find use in marker assisted selection (MAS) programs. This type of selection causes the genetic information of markers to be used as an indirect selection criterion for the genetic improvement of a quantitative trait. Summing up, it can be stated that the results of the conducted own research can be used in the selection of cattle and translate into economic benefits for cattle breeders. Certainly, these results should be treated as preliminary due to the small number of animals included in the experiment, and the next step should involve extension of the research to other breeds of cattle and more numerous herds of animals.

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Statement of conflicts of interest

The authors have declared no conflict of interest.

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