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The Effects of Negative Pressure on the Gene Expression of Motility Related Proteins in Boar Spermatozoa during Liquid Storage at 17°C

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ABSTRACT

Background: The present study was aimed to investigate the effects of negative pressure applied before storage on the gene expression of motility related proteins in boar spermatozoa.

Methods: Boar semen samples were collected and pooled and diluted with Modena solution containing 0.4% (w/v) of bovine serum albumin. Negative pressure was applied for 2-5 min using a vacuum pump with a barometer. The pressure applied was 0 (Control), -0.02 MPa (P2), -0.04 MPa (P4) and -0.08 MPa (P8). The expression of AQN-1, AQN-3, AWN, PSP-I, PSP-II gene in boar spermatozoa was evaluated.

Result: Application of -0.04 MPa improved the sperm motility compared with the other groups. In conclusion, our results confirmed thatnegative pressure preservation at 17°C had an effect on the expression of boar sperm adhesion protein gene. The relative expression of AWN0PSP-I and PSP-II genes in boar sperm were lower under -0.04 MPa and -0.08 MPa pressure, which was beneficial to protect spermatozoa motility.

Key words: Boar semen, Gene expression, Motility related proteins, Negative pressure.

INTRODUCTION

The expression of related protein genes is closely related to the quality of sperm during the *sperm development* in testes, storage in epididymis and preservation in vitro. The sperm adhesion protein gene family plays an important role in the process of spermatogenesis, ejaculation and sperm-egg binding. AQN-1, AQN-3, AWN, PSP-I, PSP-II adhesion protein gene were the main member of sperm adhesion protein gene family. The sperm adhesion protein gene family had a high abundance of mRNA expression in boar spermatozoa (Gao *et al*., 2014). AQN-1 and AWN prevent premature sperm capacitation and AQN-1 is involved in the formation of sperm banks. AQN-3 is related to the formation of the sperm motility inhibitor and can promote the capacitation of the sperm after the combination with AWN. The PSP-I/ PSP-II complex and AQN-1 binding can protect the integrity of the sperm membrane.PSP-I and PSP-II are also associated with the maintenance of sperm motility and mitochondrial activity (Caballero *et al.*, 2006).

Some researchers have been studied on the quality of semen storage and the expression of related genes. The level of expression of mRNA of the sperm is associated with the viability and availability of the spermatozoa (Ding *et al*., 2016). The GAO's results (2014) showed the same existence of a wide range of RNA molecules, whose functions involve nucleic acid binding, structural modification and transcriptional regulation (Gao *et al*., 2014). The sperm adhesion protein gene family has a high abundance of mRNA expression in boar spermatozoa (Gao *et al*., 2014). The family of the sperm adhesion protein is expressed in the reproductive tract of the male animal, which is also an important part of the seminal plasma protein. AQN-3 is involved in the formation of the sperm motility inhibitor complex. In the process of ejaculation, the AWN protein and AQN-3 protein in the seminal plasma showed the characteristics of the combination with the phospholipid. And the AQN-1 protein and the AWN protein are involved in the formation of the acrosomal reaction inhibition receptor complex before sperm-egg binding and the premature sperm capacitation can be prevented by them (Lehti *et al*., 2017). In addition, AWN and AQN-3 also mediate sperm binding to

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the pellucida during fertilization (Verónica *et al*., 2014). PSP-I and PSP-II are the most abundant proteins in seminal plasma. They can form heterodimer and have many functional properties. PSP-I and PSP- II have a special CUB domain like the expressed protein of AQN1, AQN3, AWN.

Some studies have shown that PSP-I and PSP-II genes are also expressed during sperm capacitation. PSP-I/ PSP-II complex and AQN-1 protein can act on acrosome (Caballero *et al*., 2006) and protect sperm membrane integrity, motility and mitochondrial activity (Caballero *et al*., 2004). However, the stability of PSP-I protein and PSP-II protein are decreased in acidic environment. When spermatozoa is in the female reproductive tract, pig seminal plasma protein can control the entry of white blood cells (Washington *et al*., 1997; Centurion *et al*., 2003). Basioura (2018) and Gadea (2004) have confirmed that the PSP-I/ PSP-II complex can interact with transmembrane protein MS4A8B, which acts on MS4A. The homologue of CD20 transmembrane protein was expressed by T lymphocytes through protein network analysis, which can inhibit the proliferation of T lymphocytes and B cell signal transduction (Basioura *et al*., 2018; Gadea *et al*., 2004).

In our previous study, the boar semen was stored in the negative pressure environment (-0.04 MPa,-0.08 MPa) and the vitality and the quality of the sperm were better than those stored under normal atmospheric pressure (Li *et al*., 2018; 2019). At the same time of improving its quality, it is possible to relate the gene and expression to the adhesion of the sperm. The purpose of this study is to determine the AQN-1, AQN-3, AWN and PSP-I/PSP-II of the sperm under 17°C, negative pressure. The relative expression of adhesion proteins gene was used to evaluate the effect of negative pressure on the expression of the sperm adhesion proteins.

MATERIALS AND METHODS

The group design was shown in Table 1. This study was conducted at the Animal Reproduction Laboratory of Heilongjiang Bayi Agricultural University in China from January 2018 to June 2019.The expression of AQN-1, AQN-3, AWN, PSP-I, PSP-II gene in spermatozoa was measured at 0, 3, 5 and 7 days of semen preservation and the experimental records were made. Each group was repeated 4 times or 5 times.

The main kit required for this experiment is shown in Table 2 and other reagents were purchased Sigma, except for special instructions. The designed primers are synthesized by Harbin Qingke biological company. The boar semen was collected from 10 boars in porcine farm of Heilongjiang Bayi Agricultural University.

Preparation of spermatozoa

1.5 mL of semen was separately collected and centrifuged at 1500 rpm for 5 min from each group. The supernatant was removed and the pellet was washed three-times by centrifugations at 1800 rpm for 5 min with PBS. After washing, the pellet was re-suspended with 1.5 mL of PBS, which was centrifuged at 12,000 rpm for 1 min. The precipitate was re-suspended with 200 µL of TRIzol, which was quickly frozen by the liquid nitrogen until use.

Table 1: Group designs.

Group	Control	P2	P4	P8
Pressure	Normal atmospheric pressure	-0.02 MPa	-0.04 MPa	-0.08 MPa

Table 2: Main reagents or kits.

Appellation	Factory	Serial number
Prime script™ RT reagent Kit with gDNA eraser	Takara company	RR047A
TB green™ Premix Ex Taq™	Takara company	RR420A

RNA extraction

The frozen semen was collected and ground by the electric grinding rod. The liquid nitrogen was continuously replenished during the grinding process and the mixture was ground for 3-5 times and then 800 µL of TRIzol was added and the solution was repeatedly pumped by using a 1-mL syringe until the solution has no obvious floccules. And then the operation steps are the same as the extraction of the total RNA of the normal cells.

The synthesis of cDNA

The synthesis of cDNA was carried out in accordance with the instructions on the cDNA reverse transcription kit.

Real-time fluorescence quantitative polymerase chain reaction (PCR) detection

Each 1 µg of total RNA from the different treatment groups was reversely transcribed into cDNA and the cDNA was diluted 10-fold with ddH_2O. According to the sequence of known genes, the gene-specific primers (Table 3) and GAPDH are the internal reference genes. The expression of the sperm adhesion protein gene in different treatment groups was detected by fluorescence quantitative PCR. The TB Green system was used to: DNA template (<100 ng) 2µL, TB Green Premix Ex Taq 10 µL, PCR Forward Primer (10 µM) 0.5 µL, PCR Reverse Primer (10 µM) 0.5 µL, ddH_2O to 20 µL.

The reaction conditions were detected by fluorescence quantitative PCR: 95°C 30 s, 95°C for 5s, 58°C for annealing and 30 s for extension and 40 cycles. And the relative expression level of the boar sperm adhesion protein gene was analyzed by using GAPDH as the internal reference gene.

Statistical analysis

Data from four replicated trials were analyzed by one-way ANOVA using the STATVIEW 5.0 software (Abacus Concepts, Inc., Berkeley, CA, USA). If the P value was smaller than 0.05 in ANOVA, Bonferroni/Dunn test was carried out using the same program. All data were expressed as means± SD. Findings were considered significantly different at $P<0.05$.

RESULTS AND DISCUSSION

The effect of negative pressure on the expression of AQN-1 protein in boar sperm was shown in Fig 1. The relative expression of AQN-1 gene in boar sperm was not significant (*P>0.05*) at 0,3,5,7 and 7 days of semen preservation. As shown in Fig 1, as the retention time increased, the relative expression of the AQN-1 protein gene increased. And after 5 days of storage, the relative expression level of AQN-1 protein is higher than 0 or 3 days of the storage. In this study, there was no difference in the relative expression of AQN-1 protein gene among the experimental groups. But with the increase of preservation time, the relative expression of mRNA in sperm adhesion protein gene family increased. The results of previous experiments have shown that the relative expression of mRNA in sperm adhesion protein gene family is increasing with the increase of preservation time, which is consistent with the results of GAO (GAO *et al*., 2014).

The effect of negative pressure on the expression of AQN-3 protein gene in boar spermatozoa was shown in Fig 2. There was no significant difference in the relative expression of AQN-3 protein gene in boar spermatozoa on 0 day and 3 days of preservation *(P>0.05*). After preservation for 5 days, the relative expression of AQN-3 protein gene in P4 and P8 groups was higher than that in control group and P2 group (*P<0.05*), but there was no significant difference between P4 and P8 groups (*P>0.05*) and there was no significant difference between P2 group and control group (*P>0.05*). On preservation for 7 days, the relative expression of AQN-3 protein gene in P2 or P4 group was significantly higher than control group *(P<0.05*) and there was no significant difference in the relative expression of AQN-3 protein gene between P8 group and control group *(P>0.05*). The relative expression of AQN-3 protein gene was lower than that of the control group after 3 to 5 days of preservation under -0.04 MPa. The previous results also showed that the sperm motility was the best when the preservation condition was proper, which may also be related to the low relative expression of AQN-3 protein gene (Li *et al*., 2018; 2019).

The effect of negative pressure on the expression of AWN protein gene in boar spermatozoa was shown in Fig 3. There was no significant difference in the relative expression of AWN protein gene in all groups at the 0 day and 7 days of preservation (*P>0.05*). The relative expression of AWN protein gene in P2 group and control group was significantly higher than P4 and P8 groups on the 3 day after preservation, but there was no significant difference between

Table 3: Primers used for quantitative real time PCR.

Gene	Primer sequence (5′-3′)	Gen bank no.	Fragment size (bp)
GAPDH-F	CTCTGCACGTCAGCGATCTA	AF017079	346
GAPDH-R	AACGTCCGGATTGGGTAGAG		
AQN1-F	CTTCTTCCAATGCCTTGAGC	NM_001025210	214
AQN1-R	GAGGTTGGGGTGGGAGTATT		
AQN3-F	GGTTTGTGGAGGCACAACTT	NM_001031776	210
AQN3-R	GCCTGCTGATTCCAGAAGAG		
AWN-F	GTGCCTCTCCCTTCCACATA	NM_213829	180
AWN-R	CAGGGACGAGAGGATGTGAT		
PSP-I-F	GGAGCCCCAGGATCTAAGAG	NM_213837	211
PSP-I-R	GTGGAAGATCACGCTCATCA		
PSP-II-F	AATCTGTGCACCTGGACCAT	NM_213836	163
PSP-II-R	AAAAGACAATCGCTGCACCT		

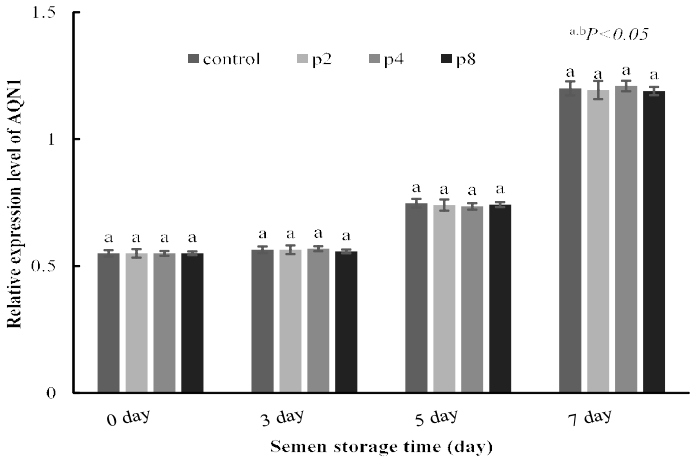

Fig 1: Relative expression level of AQN-1.

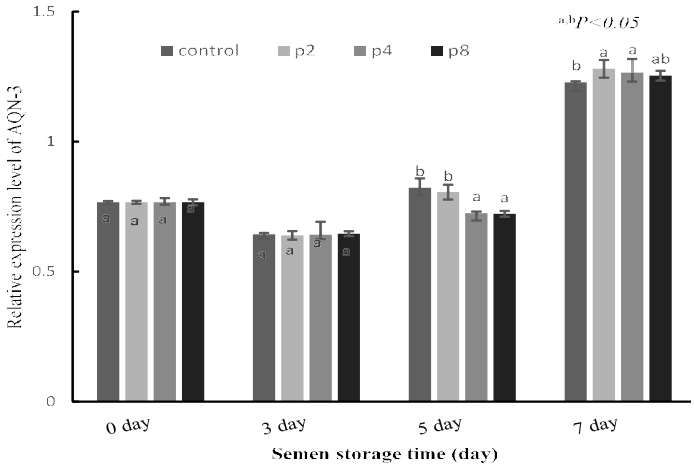

Fig 2: Relative expression level of AQN-3.

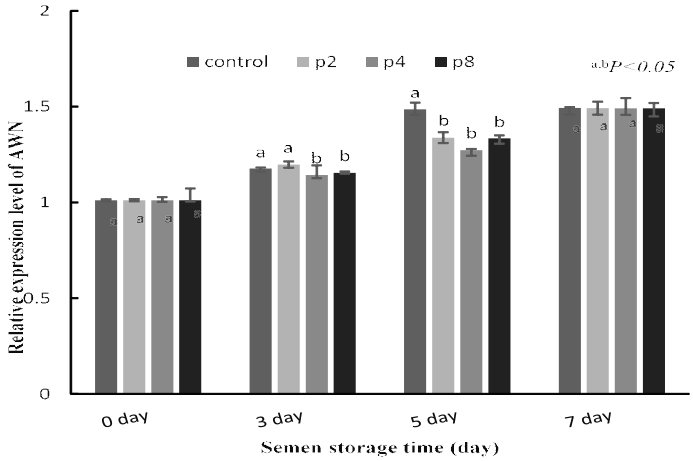

Fig 3: Relative expression level of AWN.

P2 group and control group and there was no significant difference between P4 and P8 group. On the 5 days of preservation, the relative expression of AWN protein gene in the control group was significantly higher than the other groups (*P<0.05*). This results showed that the relative expression of AWN, PSP-I and PSP-II genes in negative pressure group was lower than control group. The expression of AQN-3 and AWN protein genes of negative pressure preservation group was higher than control group at 7 days after preservation, which may be related to the proportion of dead spermatozoa. It can be seen from the results of the previous experiment that the sperm motility of control group after preservation 7 days was lower than that negative pressure group, but the specific reasons need to be further studied.

The effect of negative pressure on the expression of PSP-I and PSP-II protein in boar spermatozoa was shown in Fig 4 and 5. There was no significant difference in the relative expression of PSP-I/ PSP-II *(P>0.05*) in all groups on 0 and 3 days of preservation (*P>0.05*). The relative expression of PSP-I gene in the control group was significantly higher than the other groups (*P<0.05*) on the 5 days of preservation, but there was no significant difference in P2, P4 and P8 groups (*P>0.05*). On the 7 days of preservation, the relative expression of PSP-I/ PSP-II protein gene in the control group was significantly higher than the other groups (*P<0.05*), however, not significant in P4 and P8 groups (*P>0.05*). Centurion *et al*. (2003) showed that the porcine sperm adhesion protein gene family was involved in the regulation of sperm motility and the heterodimers formed by PSP-I protein and PSP-II protein could bind to heparin. Carcia *et al.* (2006) found that the main functional group of PSP-I/PSP-II dimer in inhibiting sperm motility was the subunit structure of PSP-I and the ability of single subunit to inhibit sperm motility was better than that of PSP-I/PSP-II dimer. At the same time, AQN-3 may be a member of boar sperm motility inhibitor complex (Shultz *et al*., 2006). Song *et al.* found that the mRNA expression level of adhesion protein gene family in capacitated sperm was significantly higher than infertile sperm. The results of GAO (2014) showed that the expression of adhesion protein gene family mRNA in low motility spermatozoa was significantly higher than that in high motility spermatozoa (GAO *et al*., 2014).

Although mRNA of boar sperm has been widely reported, most studies have focused on the types of RNA, the structure of the expressed products. However, there are

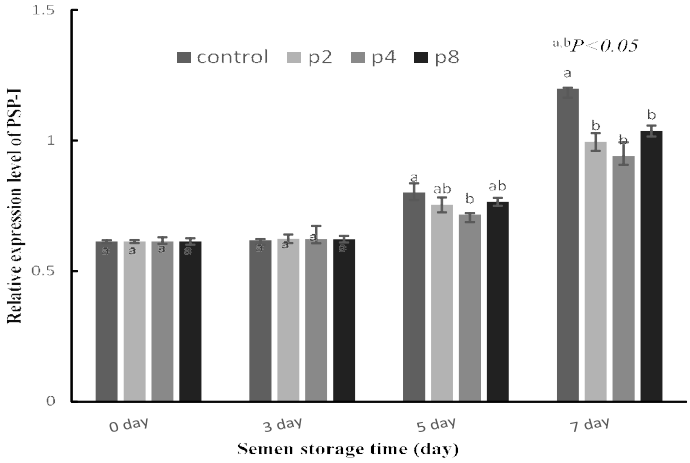

Fig 4: Relative expression level of PSP-I.

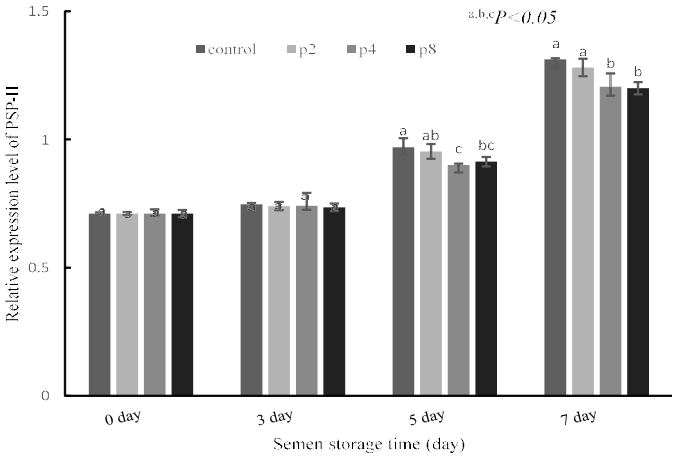

Fig 5: Relative expression level of PSP-II.

few studies on its biological function and mechanism and the biological function and mechanism of many sperm RNA still need to be studied. Some studies found that AQN-1, AQN-3 and AWN are related to sperm capacitation (Lambard *et al*., 2004; Lalrintluanga, *et al*., 2014). GAO reported that the expression of AQN-1, AQN-3, AWN, PSP-I and PSP-II genes in pig spermatozoa was negatively correlated with sperm motility (GAO *et al*., 2014).

CONCLUSION

Negative pressure preservation at 17°C has an effect on the expression of boar sperm adhesion protein gene. Overall, the relative expression of porcine sperm AWN0PSP-I and PSP-II genes was low under -0.04 MPa and -0.08 MPa pressure, which was beneficial to protect sperm motility.

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