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Research Article

Effect of NAGase on sperm motility related to Izumo4 protein *in vitro*

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Abstract

N-Acetylglucosaminidase (NAGase) is an enzyme found in the sperm acrosome of numerous animal species and it has a high activity in both eggs and sperms. The present study shows the potency of NAGase in sperm motility. In pig sperm, the sperm acrosomal integrity, mitochondrial activity and motility parameters (forward motion, cephalic swing and linear velocity) were observed in the presence of acetamide, an inhibitor for NAGase. They were significantly (P<0.01) decreased with the acetamide treatment. Importantly, the differential protein of sperm was screened in the presence of acetamide. These protein includes phosphatidylethanolamine binding protein 4 (PEBP4), Cytochrome c oxidase α-subunit polypeptide 1, Izumo sperm-egg fusion protein 4 (Izumo4), dihydrolipoic acid dehydrogenase protein, TCP1 chaperone protein, spermplasma glycoprotein PSP-I, pig sperm adhesion protein AWN-1 and sugar-binding protein AQN-3. Thereinto, the Izumo4 protein as the core protein metabolic network involved sperm-egg recognition and sperm capacitation by the protein interaction analyzed. Moreover, the inhibition of the activity of NAGase in sperm has negative regulatory activity on Izumo4 based on the results of the dual luciferase reporter genes. Our research proves that NAGase can play a significant role in the sperm function associated with the fertilization process.

Key words: Izumo4 protein, Motility, NAGase, Sperm

Highlight

- Sperm acrosomal integrity, mitochondrial activity and motility parameters were significantly decreased with acetamide treatment.
- NAGase in sperm was negative regulatory activity on Izumo4 protein.

INTRODUCTION

N-acetylglucosaminidase (NAGase) has been repetitively implicated in fertilization by glycosidase-recognizing N-acetylglucosamine terminal residue and they play a role in molecular recognition and bioinformatics transmission in mammals and insect (Cattaneo *et al*., 2002; Wang and Hanover, 2013; Stephens *et al*., 2015). Especially, in reproduction process, NAGase participate the initial stage of sperm-egg recognition through zona pellucida in human and mice (Koyanagi and Honegger, 2003; Rodeheffer and Shur, 2004; Zitta *et al*., 2006; Petit *et al*., 2013; Sarosiek *et al*., 2018). Previous studies on the expression of NAGase in the reproductive system of different animals indicated that NAGase was expressed in spermary and sperm cells (Godknecht and Honegger, 1991; Perez Martinez *et al*., 2008; Zhang *et al*., 2014; Stephens *et al*., 2015; Sarosiek *et al*., 2018; Sun *et al*., 2018). Importantly, the expression level of NAGase in pig semen is higher than that of chicken, sheep and cattle (Song *et al*., 1987). Previous results showed that the ascidian viscera NAGase treatment could significantly reduce allergenicity of ovalbumin from chicken egg (Hwang *et al*., 2016; Park *et al*., 2017). In addition, the NAGase activity was correlated with sperm quality and fertilization rate (Godknecht and Honegger, 1991; Miller *et al*., 1993; Sarosiek *et al*., 2014; Sarosiek *et al*.,

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2018). In an *In vitro* experiment, the mouse sperm was treated by PUGNAC, an inhibitor of NAGase, the recognition signal of NAGase glyco chain was significantly reduced, leading to reduction in sperm-egg binding ability (Miller *et al*., 1993). In an experiment of rainbow trout sperm, it was proved that acetamide treatment could significantly reduce the sperm cell NAGase activity, thereby reducing the sperm fertilization rate from 79% to 40% (Sarosiek *et al*., 2014).

Many scholars have conducted a large number of studies on the mechanism of sperm-egg fertilization (Zitta *et al*., 2006; Sarosiek *et al*., 2014), especially in-depth understanding of the structure and function of sperm acrosome (Brandelli *et al*., 1994; Downey and Lambert, 1994; Godinez-Solis *et al*., 2019), but still many controversies exist. In present study, we analyzed the effect of acetamide on sperm NAGase enzymatic activity, mitochondrial integrity and activity of sperm, the differential proteins expression in boar sperm cells, thereby analyzed the molecular mechanism of acetamide treatment inhibiting the sperm cell NAGase activity affect the sperm function by 2-D method and the luciferase reporter. In this study, semen was treated with NAGase activity inhibitor *in vitro*, the protein expression changes in spermatozoa were analyzed, the differential proteins were screened, and the relationship between NAGase interacting proteins and spermatozoa was studied, which laid a foundation for further research on the regulation of sperm physiological function by NAGase, and provided new ideas for clinical application in animal breeding.

MATERIALS AND METHODS

Preparation of sample: The pig sperm samples were collected from the breeding pig farm (Fuqing, China) by performing a few cuts in epididymis with micro-dissection scissors. The animals were kept at the Fujian Agriculture and Forestry University Animal Facility, and used in accordance to the guidline of the Fujian Agriculture and Forestry University Institutional Animals Care and Use Committee. In order to maintain the activity of boar sperm, sperm were collected in M199 medium containing 0.4% BSA and incubated in 5% CO_2 incubator at 37°C for 1 h for keeping them active.

Isolation and purification of semen NAGase: 600 mL of the semen sample prepared above was frozen and thawed repeatedly in the refrigerator at -80ºC and the sperm cells were crushed by ultrasound. The semen was filtered by clean gauze to obtain 500 mL seminal plasma. The 0.01 mol/L Tris-HCl buffer (pH=5.7) with containing 0.2 mol/L NaCl was placed in 4ºC refrigerator for pre-cooling for 2 h, and then added into the seminal plasma at a ratio of 1:1 (V/W). After 12 h at 4ºC, the supernatant was centrifuged at 10000g at 4ºC for 25 min. The solid ammonium sulfate was prepared into powder and mixed with the supernatant until the saturation of ammonium sulfate in the supernatant reached 35%. The supernatant was incubated at 4ºC for 4 h and then centrifuged at 4ºC at 10000g for 25 min. The supernatant was collected and ammonium sulfate powder was added to the supernatant until the saturation of ammonium sulfate reached 75%. The supernatant was discarded to collect sediment. The precipitate was dissolved in 10 mL of 0.01 mol/L Tris-HCl (pH 5.7) buffer. The solution was placed in a pre-soaked dialysis bag and dialyzed until no SO_4^{2-} was detected. Then the dialysate was placed at 8000 g, centrifuged at 4ºC for 25 min, and the supernatant was retained to obtain crude enzyme. NAGase was separated from crude enzymes by DEAE cellulose filtration gel column chromatography, CM Sepharose Fast Flow gel column chromatography and enzyme concentrated Sephadex G-100 filtration gel column chromatography.

Sperm function analysis: The sperm motility was assayed by the computer-assisted sperm quality analysis system according to the previous research (Huo and Yang, 2000; Ryu *et al*., 2014). The 50 mL sperm suspension was

put into a 50 mL EP tube and placed in a 37ºC water bath for about 45 min. After that, about 1/3 of the sperm suspension was absorbed, and an equal volume of sperm diluents was added, and then gently mixed up and down. The 4 mL spermatozoa were divided into glass tubes with high temperature sterilization, clean and preheated at 37ºC. The different concentrations (15, 50, 100 and 200 mmol/L) acetamide was used and treated with the sperm. After incubation for 15, 30 and 60 min, the integrity of the acrosomal sperm and the forward motion, cephalic swing and linear velocity of sperm was analysis by HT-CASA-system, and the sperm mitochondrial activity was analyzed by Rhodamine 123 (Eugene, Molecular Probes) and PI staining (Garner *et al*., 1997).

Protein sample preparation and 2-DE: All chemicals used for protein separation and extraction were of analytical grade. For the proteomic analysis, pig spermatozoa samples were weighed and homogenized in an ice-cold buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 50 mM DTT, 1 mM PMSF and 0.8% (v/v) biolytes (Bio-Rad) (Jiang *et al*., 2013). The homogenates were swirled for 30 min, followed by a 30-min centrifugation at 15000×g at 4°C. The supernatant containing the total sperm protein content was collected and the protein concentration of the sample was quantified using the Bradford method (Candiano *et al*., 2004). Approximately 960 µg total proteins were separated by loading the sample on a 17-cm pH 3-10 nonlinear gradient IPG strip (Bio-Rad). The strip was subjected to IEF with a voltage gradient of 250 V for 1 h, 500 V for 1 h, 2000 V for 1 h, and 8000 V for 3 h, followed by holding at 8000 V until a total of at least 60 000 V-h was reached. The second electrophoretic dimension was 12% SDS-PAGE (Jiang *et al*., 2013). The protein signal was visualized by G-250 (CBB). The gel image was digitalized with a gel scanner (Power-look 2100XL, UMAX), and analyzed using Version 8.0 PDQuest™ software package (Bio-Rad). We compared the abundance differences between HCD (treated) and LCD (control) samples by three biological repeats to find the differentially expressed protein spots. For each spot, the mean relative volume (RV) was changed more than 2.0-fold or less than 0.5-fold and $P<0.05$ was considered differentially expressed.

In-gel digestion and MALDI-TOF-TOF analysis: Differentially expressed protein spots were excised from gels, washed with Millipore pure water for three times. In-gel digestion was performed and the peptides were extracted twice with 0.1% TFA in 50% acetonitrile. Extracts were pooled and lyophilized. The resulting lyophilized tryptic peptides were dissolved in 5 mg/mL CHCA containing 0.1% TFA and 50% acetonitrile. MALDI-TOF-TOF MS analysis was conducted using 4800 Plus MALDI-TOF-TOF™ analyzer (Applied Biosystems, Foster City, CA, USA). All spectra of proteins were submitted to database searching using online MASCOT program (http://www.matrixscience.com), against NCBInr databases. Protein significant hits, as defined by the MASCOT probability analysis ($P<0.05$), was considered acceptable.

Luciferase reporter assays: A full-length overlap primer was designed. The IZUMO4 promoter region and the transcription factor NAGase sequence were synthesized by two rounds of PCR, which were respectively linked into pGL3-basic and pcDNA3.1 (+) vectors to obtain the recombinant vector of IZUMO4 promoter and NAGase. A total of 293FT cells were transfected with Lipo3000 liposome (Invitrogen, L3000015) following the manufacturer's protocol. The cells were subjected to further experimentation at least 48 h after transfection. Light switch luciferase assay reagents were obtained from Promega (Corporation, USA). The IZUMO4 over-expressing 293FT was transfected together with a luciferase reporter plasmid and the psiCHECK™-2 vector expressing Renilla luciferase for 24 h. Luciferase activity was measured using a dual reporter assay system

according to the manufacturer's instructions. Renilla luciferase was used for normalization.

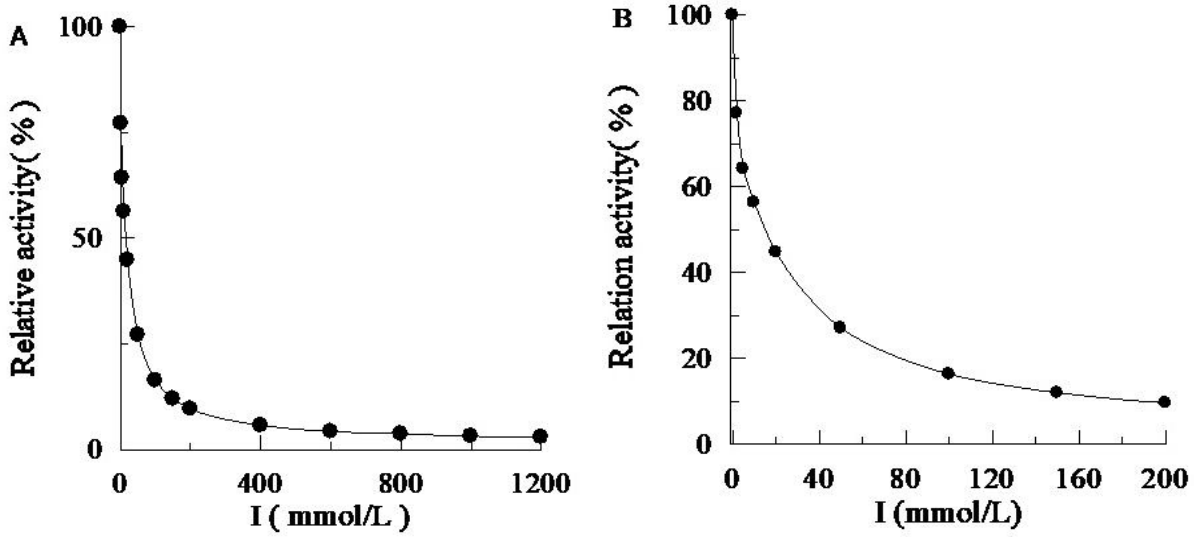
Statistical analysis: The one-way analysis of variance (ANOVA, Statistical Packages for Social Science 20.0) was used to analyze the data by a multiple range test. A replicate was considered the experimental unit for the entire index determined. The results were expressed as the mean ± SEM. $P<0.05$ or $P<0.01$ shows the significant or extremely significant differences.

RESULTS

Effects of acetamide on NAGase activities: As shown in Fig. 1, the relative activity of the enzyme decreased rapidly with the increase of acetamide concentration in the range of 0-200 mmol/L. The concentration of acetamide decreased by 50% to 15 mmol/L (IC50). In the range of 200-1200 mmol/L, the inhibition of acetamide on the enzyme tended to be gentle.

Effects of acetamide on sperm function: As shown in Table 1, pig semen treated with acetamide at different concentrations (15, 50, 100 and 200 mmol/L) showed that the activity of NAGase was inhibited *in vitro* incubation acetamide for 15, 30, and 60 min and the integrity of the sperm acrosomal was also significantly ($P<0.01$) reduced. The results of 15, 50 and 100 mmol/L acetamide *in vitro* incubation for 15 min showed that the acrosomal integrity of sperm was not difference with the increase of acetamide concentration. However, the activity of NAGase and acrosomal integrity of sperm were significantly decreased ($P<0.01$) *in vitro* incubation for 15 min, 30 min and 60 min when the concentration of acetamide was 200 mmol/L.

As shown in Table 2, sperm mitochondrial activity was significantly reduced with the different concentrations (15, 50, 100 and 200 mmol/L) of acetamide treatment *in vitro* for 15, 30 and 60 min ($P<0.05$). During the same incubation time, the results of acetamide treatment of pig semen showed that acetamide inhibition the sperm mitochondrial activity has dose-dependence. With the increase of acetamide concentration, sperm mitochondrial activity was decreased. When the concentration of acetamide was greater than 100 mmol/L, the sperm mitochondrial activity was significantly ($P<0.01$) inhibited.



[(A) Acetamide concentrations were 0-1200 mmol/L, (B) Acetamide concentrations were 0-200mmol/L]

Fig. 1. Effects of acetamide on the activity of NAGase

Table 1. Effect of different concentrations of acetamide on acrosome integrity of sperm by inhibiting NAGase activity

Added acetamide concentration (mmol/L)	15 min	30 min	60 min
0	97.23±0.85^{Aa}	94.33±1.02^{Aa}	90.26±0.55^{Aa}
15	82.46±1.20^{Bb}	77.60±0.45^{Bb}	71.36±0.86^{BDb}
50	81.26±1.30^{Bb}	76.46±0.21^{Bbc}	68.56±1.25^{BCDc}
100	80.80±1.25^{BCb}	75.33±0.21^{Bc}	66.86±2.08^{CDc}
200	78.20±0.65^{Cc}	72.60±1.77^{Cd}	68.26±1.02^{Dc}

Note: In the same column, different uppercase letters of shoulder caps superscripts showed significant differences between groups (P<0.01); Small letters with different shoulder caps superscripts indicated significant differences between groups (P<0.05); Uppercase or lowercase letters of the same shoulder mark indicated no significant difference between groups (P>0.05).

Table 2. Effect of different concentrations of acetamide on sperm mitochondrial activity by inhibiting NAGase activity

Added acetamide concentration (mmol/L)	15 min	30 min	60 min
0	91.0±1.50^{Aa}	86.8±2.84^{Aa}	83.0±3.96^{Aa}
15	57.5±3.00^{Bb}	52.8±1.25^{Bb}	48.2±3.96^{Bb}
50	56.3±2.08^{Bb}	49.3±1.52^{BCbc}	43.8±0.76^{BCbc}
100	53.5±5.34^{BCbc}	46.2±2.46^{Ccd}	43.7±0.57^{BCc}
200	48.8±5.34^{Cc}	43.8±3.68^{Cd}	39.5±3.04^{Cc}

Note: In the same column, different uppercase letters of shoulder caps superscripts showed significant differences between groups (P<0.01); Small letters with different shoulder caps superscripts indicated significant differences between groups (P<0.05); Uppercase or lowercase letters of the same shoulder mark indicated no significant difference between groups (P>0.05).

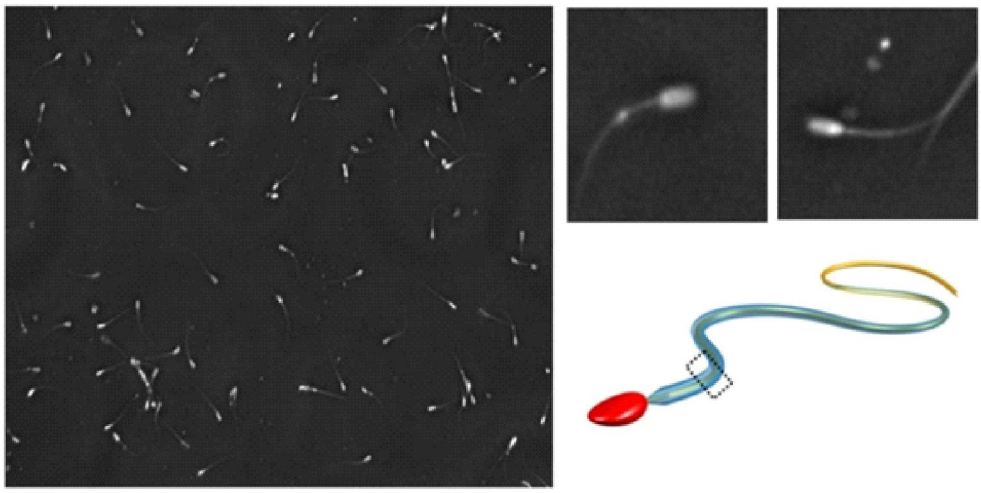


Fig. 2. The Sperm motility capture diagram and motility model

As shown in Fig. 2, the movement of sperm mainly depends on the swing of the tail, and the capture results showed that the movement performance of sperm was normal in the control group. After 60 min incubation with different concentrations (15, 50, 100 and 200 mmol/L)

acetamide, the forward motion, cephalic swing and linear velocity of sperm were significantly decreased compared with those of control group ($P<0.05$ or $P<0.01$, Table 3). In addition, the motility percentage of pig semen treated with different concentrations of acetamide (15, 50, 100 and 200 mmol/L) was decreased after 60 min *in vitro* incubation to inhibit the activity of NAGase enzyme.

Effects of acetamide on sperm function associated with target protein: The results of 2-D electrophoresis showed that the sperm protein samples were separated by 12.5% SDS-polyacrylamide gel with 24 cm adhesive strips at pH 3-10. After silver staining, 57 differentially expressed protein spots were detected in the gel, and the protein spots were mainly distributed in the range of pH 3-10 and relative molecular weight 14.4-116 kDa. According to the screening results of differentially expressed proteins, representative maps from the three repeated experiment results were selected for labeling, and protein spots with up-regulated expression levels in different comparison groups were labeled on the corresponding 2-DE maps (Fig. 3). Under the same experimental conditions, two dimensional electrophoresis results were analyzed by PDQuest 8.0 software and spot matching was performed. After manual point-by-point calibration for quality inspection, the average matching rate was more than 82.7%. Differential protein spots were separated from the gel and digested. The digested protein samples were analyzed by mass spectrometry for differential protein identification. The number of differentially expressed proteins screened in each comparison group was as follows: 45 differentially expressed proteins in the control group and 12 differentially expressed proteins in the experimental group. Importantly, nine typical differential proteins related to sperm function were successfully identified by NCBI and MASCOT database, two of which were identical proteins. The screened differential protein relative molecular weight (mRS) and isoelectric point (pIS) were close to the theoretical values. Among the 8 matched proteins were selected including phosphatidylethanolamine binding protein 4(PEBP4), Cytochrome c oxidase Vl α-subunit polypeptide 1(COX6A1), Izumo sperm-egg fusion protein 4 (Izumo 4), Dihydrolipoic acid dehydrogenase (DLD) protein, TCP1 chaperone protein, spermatoplasma glycoprotein PSP-I, pig sperm adhesion protein AWN-1 and sugar-binding protein AQN-3

The functions of these differential expressed proteins are mainly as follows: (1) Phosphatidylenolamine-binding protein 4 (PEBP4) is mainly involved in Mitochondrial translation termination process, and in Mitochondrial ribosomal protein (MRP) -L4, MRP-L17, MRP-L44 and Death associated protein (DAP-3). For sperm cells, the up-regulated expression of this protein can reduce the ATP provided by sperm mitochondria, thus reducing sperm motility. (2) Cytochrome c oxidase α-subunit polypeptide 1(COX6A1) is mainly involved in proton transmembrane transport and mitochondrial ATP metabolism, and the proteins involved include Cox subunit 4 isoform 1 (mitochondrial), Cox subunit 7C (mitochondrial) and Cox subunit 5B (mitochondrial). (3) Izumo protein 4 is mainly involved in the process of sperm-egg recognition and fertilization, and the core lies in the plasma membrane fusion of sperm and egg to participate in single fertilization. The Izumo protein mainly includes Izumo 1, 2, 3 and 4, testis-specific serine kinase 6, CD9 antigen and ADAM metallopeptidase domain 32. The inhibition of NAGase may affect the synergistic effect of NAGase and Izumo. In addition, Izumo is closely related to the nuclear pore complex protein (ZUP). (4) Dihydrolipoic acid dehydrogenase (DLD) protein is mainly involved in the tricarboxylic acid cycle of mitochondrial energy metabolism. (5) TCP1 chaperone protein is mainly involved in the interaction between the extracellular vesicles of oviduct and sperm. (6) Sperm glycoprotein PSP-I is mainly involved in the physiological processes such as sperm capacitation and single sperm fertilization, and it interacts with acrosomal reactive binding protein (ACBP-1) and zona pellucida binding

Table 3. Effect of different concentrations of acetamide on sperm motility by inhibiting NAGase activity

Time (min)	Added acetamide concentration (mmol/L)	VCL (um/s)	VSL (um/s)	MOT (%)	PRG (%)	ALH (um)	STR (um/s)	VAP (um/s)	BCF(Hz)
15	(A, group) 0	79.12±2.66^{Aa}	50.71±1.28^{Aa}	89.98±0.21^{A}	51.06±0.94^{Aa}	3.91±0.09^{Aa}	90.79±0.90^{A}	56.88±2.51^{Aa}	29.87±0.61^{Aa}
	(B, group) 15	66.09±3.05^{Bcd}	42.24±0.97^{Bcd}	86.20±1.67^{B}	31.60±0.51^{Bb}	3.20±0.20^{Bb}	86.67±0.39^{B}	46.98±1.74^{Bc}	27.05±0.53^{Bb}
	(C, group) 50	62.98±0.95^{Bd}	41.66±2.13^{Bd}	84.83±0.81^{B}	30.73±0.75^{Bb}	3.17±..12^{Bb}	81.94±1.53^{C}	45.76±2.01^{Bc}	26.76±0.61^{Bb}
	(D, group) 100	60.26±2.97^{Bd}	39.42±2.28^{Bd}	80.73±0.77^{C}	26.80±1.04^{Cc}	2.94±0.01^{BCc}	81.00±0.86^{C}	43.20±2.44^{Bc}	24.96±0.28^{Cc}
	(E, group) 200	59.35±5.86^{Bb}	39.14±1.14^{Bbd}	78.00±1.03^{D}	25.66±0.30^{Cc}	2.89±0.02^{Cc}	76.18±0.99^{D}	43.20±0.85^{Bb}	22.55±0.47^{Dd}
30	(A, group) 0	78.28±2.56^{Aa}	50.30±1.06^{Aa}	88.36±1.88^{A}	50.23±0.66^{A}	3.67±0.12^{Aa}	90.52±0.43^{A}	56.21±2.04^{Aa}	29.21±0.10^{Aa}
	(B, group) 15	54.78±2.31 ^{Bb}	35.51±2.06^{Cc}	83.46±0.73^{B}	22.26±1.19^{B}	2.81±0.13^{Bb}	76.05±0.74^{B}	40.64±1.49^{Cc}	25.90±0.87^{Bb}
	(C, group) 50	52.54±3.27 ^{Bb}	33.66±1.43^{Ccd}	81.83±0.56^{B}	21.63±0.25^{B}	2.61±0.28^{Bbc}	73.53±1.27^{C}	36.69±1.85^{Cd}	24.52±0.86^{Bb}
	(D, group) 100	51.83±2.37^{Bb}	31.57±0.32^{Cd}	79.60±1.15^{B}	19.86±0.25^{C}	2.42±0.11^{Cc}	71.35±0.68^{D}	35.89±0.86^{Cd}	22.85±0.70^{Cc}
	(E, group) 200	51.39±2.94 ^{Bb}	31.53±2.23^{Bbd}	72.16±1.82^{C}	17.10±0.70^{D}	2.38±0.05^{Cc}	54.83±0.54^{E}	34.71±2.07^{BCbd}	20.34±0.65^{Dd}
60	(A, group) 0	77.55±2.02^{Aa}	49.89±0.84^{Aa}	88.16±0.41^{A}	49.46±0.76^{A}	3.39±0.07^{Aa}	89.91±0.53^{A}	54.75±2.57^{Aa}	29.11±0.91^{Aa}
	(B, group) 15	53.78±2.45^{Bbd}	31.78±1.14^{Cc}	81.16±1.25^{B}	20.93±0.91^{B}	2.56±0.02^{Bb}	70.88±1.39^{B}	36.93±2.10^{Bb}	24.48±0.37^{Bb}
	(C, group) 50	50.84±2.43^{Bbcd}	31.18±0.70^{CDcd}	77.66±0.91^{C}	18.93±0.58^{C}	2.34±0.05^{Cc}	67.93±1.39^{C}	35.07±2.03^{Bb}	22.59±0.49^{Cc}
	(D, group) 100	50.22±3.25^{Bd}	29.85±0.67^{Dd}	74.96±0.49^{D}	17.80±0.72^{C}	2.31±0.04^{Cc}	64.44±0.87^{D}	33.77±1.55^{Bb}	21.61±0.51^{Cc}
	(E, group) 200	48.87±1.46^{Bcd}	28.84±0.24^{BDbd}	68.53±0.49^{E}	16.06±0.47^{D}	2.17±0.04^{Dd}	51.74±1.47^{E}	32.37±1.37^{Bb}	18.81±0.51^{Dd}

Note: In the same column, different uppercase letters of shoulder caps superscripts showed significant differences between groups ($P<0.01$); Small letters with different shoulder caps superscripts indicated significant differences between groups ($P<0.05$); Uppercase or lowercase letters of the same shoulder mark indicated no significant difference between groups ($P>0.05$).

protein (ZPBP-1) to prevent multiple sperms from entering eggs. (7) Sperm adhesion protein AWN belongs to the sperm adhesion protein family, which acts closely with sperm glycoprotein PSP-II and participates in sperm capacization, single sperm fertilization and other physiological processes. (8) Sugar-binding protein AQN-3 also belongs to the sperm adhesion protein family, which acts closely with sperm glycoprotein PSP-II and participates in sperm capacization, single sperm fertilization and other physiological processes. Moreover, this protein has an activity of binding phosphorylated choline and participates in the signal transmission during fertilization.

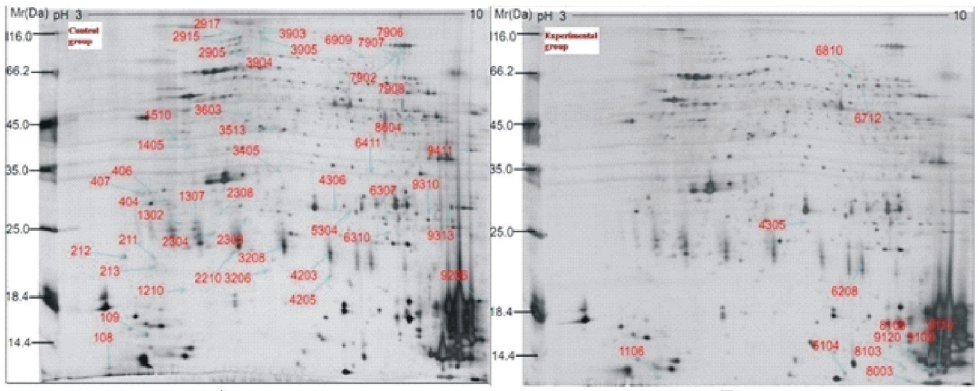
Functional analysis of differentially expressed proteins: As shown in Fig. 4A, acetamide treatment significantly increased the expression of sperm physiology-related proteins. According to KEGG database, the physiological regulatory processes involved in differential proteins can be roughly divided into 5 categories. The processes involved included spermatogenesis (11%), single sperm fertilization (44%), mitochondrial function (11%), sperm-egg recognition (11%), and mitochondrial energy supply (22%). Importantly, the differential expression protein Izumo4 was identified in this study, which mainly involved in the process of sperm-egg recognition and fertilization, and its core lies in the plasma membrane fusion between sperm and egg to participate in single fertilization. The proteins are mainly nuclear pore complex proteins including nuclear pore complex protein 93, nuclear pore complex protein 214, nuclear pore complex protein 85 and nuclear pore complex protein 210. Based on the above analysis, it was speculated that inhibition of inhibited the activity of NAGase in semen by acetamide significantly up-regulated the Izumo4 protein expression, which might be related to the synergism of Izumo and NAGase in the sperm-egg recognition process.

In order to clarify the correlation between NAGase and Izumo in the sperm-egg recognition process, we predicted the protein interaction by the String tool. The results showed that the differentially expressed proteins in sperm cells interacted with Izumo protein as the core protein metabolic network when the activity of NAGase in semen was inhibited by acetamide (Fig. 4B). As shown in Fig. 4C, the recombinant plasmid of the transcription factor NAGase was used as the transcription regulator and co-transformed with the recombinant plasmid of the promoter region of Izumo4. After transfection, the luciferase activity results showed that the transfection of NAGase recombinant plasmid significantly reduced the transcriptional activity of Izumo4 compared with the control group (pcDNA3.1 (+) group) ($P<0.001$). Based on the results of the dual luciferase reporter genes, we hypothesized that NAGase had a negative regulatory activity on Izumo4.

DISCUSSION

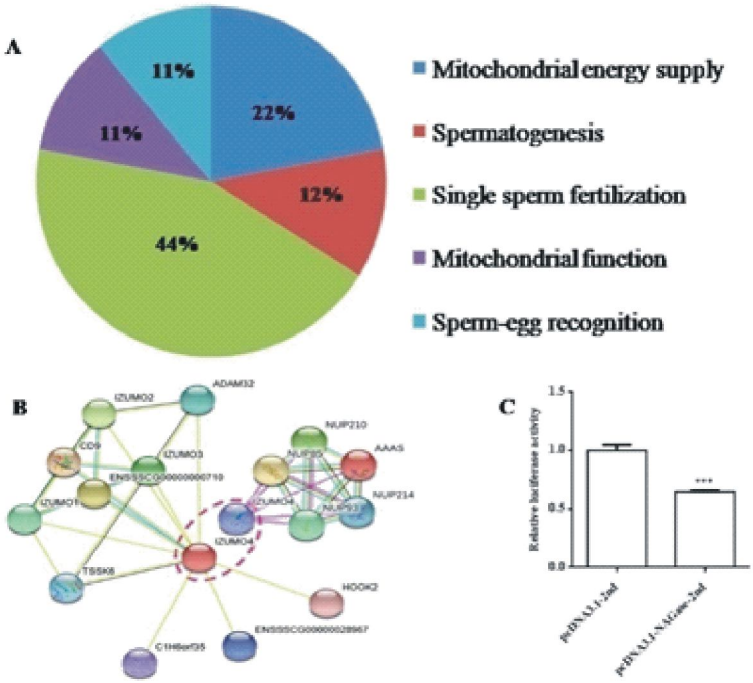
NAGase exists in the head of spermatozoa, which is very close to the acrosome, and plays an important hydrolytic activity as a hydrolytic enzyme during the acrosome reaction. The previous study indicated that abundant NAGase in the sperm tail, and the motility of sperm is closely related to its tail, which indirectly reflects that there is a certain relationship between NAGase and sperm motility (Godknecht and Honegger, 1991; Miller *et al*., 1993; Perez Martinez *et al*., 2008; Zhang *et al*., 2014; Stephens *et al*., 2015; Sarosiek *et al*., 2018; Sun *et al*., 2018). There are a large number of mitochondria at the acrosomal location, and this study showed that inhibition of NAGase can reduce the acrosomal integrity (Table 1) and mitochondrial activity of sperm (Table 2). The acrosome reaction of sperm is a crucial process in the process of mammalian sperm-egg union. Among them, acrosomal integrity of sperm is an important basis for acrosomal response (Brandelli *et al*., 1994; Downey and Lambert, 1994; Godinez-Solis *et al*., 2019). Acrosome reaction refers to the point fusion between the sperm plasma membrane and the acroextracorporeal membrane and the release

Relation between Izumo4 protein and NAGase in sperm



[(A) Control group; (B) Experimental group. Differential protein points and successful identification are numerically indicated in the 2-DE schematic diagram.]

Fig. 3. The schematic diagram of the differences expression protein spots from in sperm cells by 2-DE



[(A) Cluster analysis of differential protein; (B) STRING 9.0 analysis the protein interaction involved in the Izumo sperm-egg fusion protein 4. Different line colors represent the types of evidence used in predicting the associations: neighborhood (green), gene fusion (red), co-occurrence across genomes (blue), co-expression (black), experimental (purple), association in curated databases (light blue) or co-mentioned in PubMed abstracts (yellow). Two clusters of highly interacting protein nodes are marked with circles and include proteins involved in carbohydrate metabolism, amino acid and nitrogen metabolism, and lipid metabolism; (C) NAGase inhibits the transcriptional activity of IZUMO4, compared with pcDNA3.1-2nd group, ***P<0.001.]

Fig. 4. Functional analysis of differentially expressed protein in the sperm under NAGase protein inhibitor acetamide treatment

of acrosomal hydrolases when the capable sperm contact the egg membrane (Jin *et al*., 2011). Acrosome hydrolases are the key steps in the regulation of sperm-egg recognition and are crucial to sperm capacitation (Zhang *et al*., 2019). In addition, mitochondria, as the most important organelle in sperm cells, are different from somatic mitochondria in characteristics (Pena *et al*., 2009). Mitochondria of mammalian sperm are completely confined to the middle segment of sperm and tightly wrapped around axons to form fibrous sheaths. The activity of mitochondria in sperm is closely related to its motility, and the ability required by sperm motility is provided by its mitochondria with ATP (Pena *et al*., 2009; Losano *et al*., 2018). The sperm mitochondria are also involved in a variety of physiological processes such as sperm hyperactivation movement, capacitation, acrosomal reaction (Anifandis *et al*., 2017). Present results showed that acetamide treatment reduced the mitochondrial activity of sperm and reduced the sperm motility, such as forward motion, cephalic swing and linear velocity (Table 3). Previous result pointed out that spermatozoa movement mainly depends on the asymmetric transverse wave of flagella, which produces the rolling force caused by the unilateral force (Gadelha *et al*., 2020). The spermatozoa rotate equally in all directions to form the spiral forward movement, which promotes the forward movement of spermatozoa to facilitate the process of fertilization (Gadelha *et al*., 2020). The present results also showed that the NAGase enzymatic activity had an important role in the acrosomal integrity and sperm mitochondrial activity. With the decrease of NAGase enzymatic activity, the mitochondrial activity and acrosomal integrity was significantly decreased, thereby decreased the quality of sperm. However, the reduced forward motion of sperm may directly affect the subsequent fertilization process, which needs to be verified by subsequent studies.

Present results showed that acetamide treatment, a protein inhibitor of NAGase, significantly altered the protein expression in sperm cells by 2-D method. As shown in Fig. 4, after the activity of NAGase was inhibited by acetamide, the differentially expressed proteins in sperm cells interacted with the metabolic network with Izumo protein as the core protein, which involved the sperm-egg recognition and sperm capacitation processes. Izumo as a sperm-egg fusion protein has been proved to be a key surface protein of sperm involved in sperm-egg binding (Inoue *et al*., 2005). During sexual reproduction, a new individual is formed by the meeting and union of morphologically distinct cells produced by male and female individuals. In this process, the combination of male and female individual germ cells has a specific interaction effect, which is a process of cell membrane recognition and fusion (Stein *et al*., 2004; Liu *et al*., 2009; Klinovska *et al*., 2014). It is now known that sperm, after entering the female reproductive tract, are energized, traverse and bind to the transparent zone of the egg, triggering and completing the acrosomal response that leads to fertilization. One study showed that sperm binding on the surface of the zona pellucida of the egg cell was not sufficient to induce acrosomal responses in sperm (Gahlay *et al*., 2010). The recognition of sperm and egg depends on the division state of zona pellucid (ZP) protein, especially the division state of ZP2 glycoprotein in zonapellucida protein (Gahlay *et al*., 2010). Sperm surface ligand proteins are hidden before fertilization. In the fallopian tube, the sperm will display the ligand protein on the surface of the sperm, thereby activated the acrosomal reaction and promoted the smooth combination of sperm and egg (Okabe, 2013), which was proved to require the participation of Izumol protein and CD9 protein (Le Naour *et al*., 2000; Kaji *et al*., 2002; Liu *et al*., 2004; Tengowski, 2004; Ikawa *et al*., 2010; Liu *et al*., 2011; Claw *et al*., 2014). In a hamster model, Izumo's antibodies prevented human sperm from fusing with hamster eggs that had removed the zonapellucida (Inoue *et al*., 2005; Hayasaka *et al*., 2007). The results have also

showed that knockdown Izumo gene in the female mice has not affected the fertility and knockdown Izumo gene in the male mice also produced sperm and completed the mating behavior (Inoue *et al*., 2005; Hayasaka *et al*., 2007; Inoue *et al*., 2008; Gaikwad *et al*., 2019). However, due to the Izumo gene in the mice knockdown, the sperm could accumulate in the perioocytic space and could not produce offspring (Inoue *et al*., 2005). Previous studies have also shown that Izumo4 protein in rats and humans is located in the front of sperm head before acrosomal reaction, but they cannot be detected after acrosomal reaction (Ellerman *et al*., 2009; Singaravelu *et al*., 2015; Wang *et al*., 2015). Therefore, it is speculated that Izumo4 protein is not a transmembrane protein, which likely to be involved in sperm-egg fusion, acrosomal reaction and crossing the zonapellucida (Ellerman *et al*., 2009; Wang *et al*., 2015). Previous results indicated that the Izumo1 protein on the sperm side and its receptor Juno on the ovum side could be recognized for triggering membrane fusion (Inoue *et al*., 2015; Barbaux *et al*., 2020; Yamatoya *et al*., 2020). However, the CRISPR/Cas9-mediated mutation experiments showed that short cytoplasmic tails could regulate the expression of Izumo1, but did not participate in sperm-egg fusion (Young *et al*., 2016). The single transmembrane domain and short cytoplasmic tail of Izumo1 are expressed in the plasma membrane region of sperm after acrosome reaction, and play an important role in sperm-egg fusion, which may be related to the relatively conserved N-glycan (Satouh *et al*., 2012). These result suggested that lack of Izumo protein in sperm can inhibit the sperm function leading to fertilization failure. In the present results, the Izumo4 protein was significantly up-regulated in sperm after the inhibition of NAGase, which may indicate that Izumo4 protein is closely related to the activity of NAGase enzyme in regulating the sperm function.

Previous studies have shown that there are many proteins or key molecules involved in the plasma membrane fusion of sperm and egg, such as sperm equatorial segment protein 1 (SPESP1), cysteine-rich secretory protein 2 (CRISP2) and testis-specific serine kinase 6 (TSSK6) (Da Ros *et al*., 2004; Fujihara *et al*., 2010; Wang *et al*., 2015; Nakata *et al*., 2017). The gene knockout experiments showed that these proteins only participated in the sperm-egg fusion process, but did not play a decisive role in the process (Wolkowicz *et al*., 2008; Fujihara *et al*., 2010). According to the previous study, the structure of Izumo-1 protein involved in sperm-egg fusion is similar to that of the invading protein expression of Sporozoite microneme protein essential for cell traversal 1 (PAST1) and Thrombospondin repeat anonymous protein (TRAP) in the spore stage of Plasmodium parasites (Inoue *et al*., 2015; Barbaux *et al*., 2020; Yamatoya *et al*., 2020). It may also recruit other sperm or egg chaperone proteins in a way similar to viral fusion to form fusion proteins (Inoue, 2017). The present results showed that the expression levels of TCP1 chaperone subunit 3, sugar binding protein, plasma glycoprotein PSP-I and sperm adhesion protein AWN-1 were significantly up-regulated when the NAGase enzymatic activity was inhibited by the acetamide treatment. The previous results showed that the chaperonin-containing TCP1 complex is present on the surface of capacitated spermatozoa and activated the zona binding activity (Dun *et al*., 2011). The other results also suggested that this hydrophobic core of plasma glycoprotein PSP-I and sperm adhesion protein AWN-1 may be essential for maintaining the sperm-egg fusion process in pig (Varela *et al*., 1997; Drab *et al*., 2017). Importantly, the previous results indicated that the beta-D-N-acetylhexosaminidase could play an important role in an optimal sperm binding in pigs by modulating the amount of zona pellucida oligosaccharide moieties and their structures (Slawson *et al*., 2006; Zitta *et al*., 2006; Drab *et al*., 2017). Based on the present result, the NAGase enzymatic activity inhibition could lead to the sperm motility reduction, which may be closely related to these differentially

expressed proteins and provided the plausible explanation for how spermatozoa gain their ability to fertilize.

Based on the present results, it can be concluded that the sperm acrosomal integrity, mitochondrial activity and motility parameters (forward motion, cephalic swing and linear velocity) were reduced in the presence of acetamide, an inhibitor for beta-NAGase. Importantly, the differential expressed protein of sperm was screened in the presence of acetamide. Moreover, the inhibition of the activity of NAGase in sperm has negative regulatory activity on Izumo 4 based on the results of the dual luciferase reporter genes. Thus, it is speculated that NAGase can play a significant role in the sperm function associated with the fertilization process by regulating sperm acrosomal integrity, sperm motility and sperm Izumo4 protein interaction.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: BSL: Performed the data analyses and wrote the manuscript; QML: Contributed to the conception of the study and the financial support for conducting the experiment; GRR: Performed the experiment; ML: Contributed significantly to analysis and manuscript preparation; YHL: Contributed to the conception of the study; XHH: Helped to perform the analysis with constructive discussions.

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