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Full Length Article

The Effects of Acetamide on *N-acetyl-β-D-glucosaminidase* (*NAGase*) Activity and Sperm Motility in Semen from Boar

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Abstract

This research aimed to understand the effects of acetamide upon *N-Acetyl-β-D-glucosaminidase* (EC 3.2.1.52, NAGase) activity in semen and sperm from the boar and to investigate the relationship of *NAGase* activity with sperm motility and fertilization rate. NAGase activity from semen of the boar was determined using the kinetic method of substrate reaction to study the enzyme catalyzed hydrolysis of *p-nitrophenyl-N-acetyl-β-D-glucosaminide*. Different concentrations of acetamide were added to fresh semen and then incubated for different time periods at 37°C. Hamilton Thorne computer-assisted sperm analysis was used to test various parameters that reflect sperm motility. Results showed that acetamide dose-dependently inhibited *NAGase* activity (IC_{50}=15 mmol/L), and provided reversible noncompetitive inhibition with an inhibition constant of 2.23×10^{-2} mmol/L. The addition of 15–200 mmol/L acetamide to semen significantly decreased the percentage of progressive motile sperm, and the curvilinear, straight line and average path velocity, and lateral head displacement of sperm after incubation for 15–60 min (P<0.01). Sperm motility was significantly decreased after 60 min of incubation (P<0.05). These data suggest that acetamide had a strong inhibitory effect on semen *NAGase*, and could significantly affect sperm motility parameters. © 2019 Friends Science Publishers

Key words: Acetamide; Boar semen; *N-Acetyl-β-D-glucosaminidase*; Kinetics; Sperm motility

Introduction

Along with increasing living standards, it is a widespread mindset for the consumer, both at home and abroad, to buy superior quality and tastier pork. The fine breeding of boar provides a highly-flavored and nutrition food. Therefore, the fine breeding of boar can provide a good conomic benefit. *N-Acetyl-β-D-glucosaminidase* (EC 3.2.1.52, *NAGase*) is widely distributed in the organs of mammals, especially in pig epididymal tissue, which has high *NAGase* activity (Leaback and Walker, 1967). *NAGase* is also found in the human sperm acrosome, bull penis and boar sperm (Huang *et al.,* 2008, 2009). The enzyme is associated with animal reproduction and fertilization in species such as ascidians, fruit flies, and humans and other mammals (Miranda and Blaquer, 2000; Cattaneo *et al.,* 2002; Koyanag and Honergger, 2003). In humans, sperm *NAGase* participates in the binding to, and penetration of, egg zona pellucid (Zitta *et al.,* 2006). This enzyme plays an important role in sperm penetration through protective shields of egg (Allison and Hartree, 1970; Farooqui and Srivastava, 1980; Joyce *et al.,* 1986; Martinez *et al.,* 2000; Miranda *et al.,* 2000). *NAGase* participates in sperm capacitation, acrosome reaction and spermatozoon-oocyte interaction (Jauhiainen and Vanha-Perttula, 1986). Recently, systematic studies of *NAGase* from various animals are underway in our laboratory (Zhang *et al.,* 2012; Lai *et al.,* 2014). We have also studied the inhibitory kinetics of fructose on NAGase activity in semen of the boar.

Sperm movement characteristics and vitality are important factors in assessing sperm quality and male fertility (Sutkeviciene *et al.,* 2005; Broekhuijse *et al.,* 2011). Sperm quality is often evaluated through computer-assisted semen analysis (CASA), which is the most popular method used to evaluate sperm motility (Verstegen *et al.,* 2002; Gil *et al.,* 2009). In addition to sperm density and activity, this system can provide accurate, rapid, and objective analyses of sperm mobility and motion parameters, which include curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), and

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beat/cross frequency (BCF). Juhnke and Luedemann (1978) revealed relationships between the basic motility parameters (VAP, VSL, VCL, ALH, and BCF) and fertility. Other research found a significant correlation between sperm motility parameters (VSL, VAP, VCL, and ALH) and fertility rate (Totm *et al.,* 1991; Moore and Akhondi, 1996; Sarosiek *et al.,* 2014). CASA system is widely used in human medical for reproduction experiments, however, few researchers have applied these systems in animal experiments. In the current research, we used the Hamilton Thorne CASA system (HT-CASA-II) to quantities the effects of acetamide on pig sperm vitality and movement characteristics.

Juhnke and Luedemann (1978) revealed acetamide is a water-soluble inhibitor of *NAGase*, but does not exhibit a strong toxic effect on fish cells. Sarosiek *et al.* (2014) found that acetamide increased sperm motility parameters VAP and motility (MOT) in rainbow trout, whereas acetamide reduced sperm parameters VCL, VSL, VAP, MOT, and PRG in the Siberian sturgeon. The present study examined the effects of acetamide on *NAGase* activity and sperm motility in semen from the Duroc boar, and investigated the relationship of *NAGase* activity to sperm motility and fertilization rate. The current results have provided a technical reference for characteristics of this enzyme in swine semen, and may ultimately provide a new avenue to improve porcine breeding performance.

Materials and Methods

Biological Materials and Reagents

Biological materials: Fresh semen was provided by the boar Reservation Farm at FuJian, China. Semen samples were transported to the laboratory at 17°C within 60 min. NAGase from boar semen was purified to be PAGE homogeneous by the following techniques: $(NH_4)_2SO_4$ fractionation (40–55%), DEAE-cellulose (DE-32) ion exchange chromatography, Sephadex G-200 gel filtration and DEAE-Sephadex (A-50).

Reagents: *p-Nitrophenyl-N-acetyl-β-D-glucosaminide* (*pNP-NAG*) was purchased from the Biochemistry Laboratory of Shanghai Medicine Industry Academy (Shanghai, China). Boar semen extender (BTS) was purchased from IMV Biology Science & Technology Co. Ltd (France). Mercuric chloride ($HgCl_2$) and all other reagents were analytical grade. The water used was redistilled and ion-free.

Determination of the Inactivation Mechanism, Inactivation Type and Inhibition Kinetics

Determination of the inactivation mechanism, inactivation type and Inhibition kinetics by the concentration of acetamide (1 mol/L) was diluted to final concentrations of 5, 10, 15 and 20 mmol/L. Inhibition kinetics of boar semen *NAGase* were analyzed according to the progress-of-substrate reaction method described by Tsou (1990). All the reactions were completed at 37°C. The assay of the inactivation mechanism and inactivation type was performed according to the method of Chen *et al.* (2003) and Zhang *et al.* (2006).

Effect of Acetamide on Activity of Boar Semen *NAGase*

Enzyme activity was determined according to the method of Zhang *et al.* (2010). Different concentrations of acetamide were added to boar semen for determined *NAGase* activities. The acetamide concentration (15, 50, 100, 200, 400 and 1200 mmol/L) that reduced 50% (IC_{50}) *NAGase* activity was evaluated.

Effect of Acetamide on Sperm Motility Parameters

HT-CASA-II was used to quantify sperm motility parameters after activation to determine the effect of acetamide on boar sperm. Briefly, semen was diluted 1:1 with BTS within 10 min completed in a water bath (37°C) in the laboratory, after evaluating sperm concentration and motility. A stock concentration of acetamide (1 mol/L) was diluted to final concentrations of 15, 50, 100 and 200 mmol/L. The pH and osmotic pressure of different concentrations of acetamide were determined (Table 1). 100 μL culture fluid containing sperm (sperm counts of 18 million/mL) was added into the same volume of acetamide to obtain the final acetamide concentrations shown above. Sperm samples in the measurement system were diluted with BTS to 25×10^6 spermatozoa/mL. Three replicates were performed for each concentration of acetamide and the control sample consisted of sperm mixed with the BTS without any acetamide. A 20 μL aliquot of spermatozoa was placed on a pre-warmed glass slide after incubation for 15, 30 and 60 min, and then they were analyzed by the HT-CASA-Ⅱ system. Parameters recorded for each sample included PRG%, MOT%, ALH and BCF, movement velocity such as rapid, medium, slow, and static, and movement characteristics such as progressiveness, VAP, VSL and VCL. All trials were replicated three times. Statistical analysis Data were analyzed using *SPSS* version *19.0 software* applying one-way ANOVA.

Results

Effects of acetamide on the activity of *NAGase:* As the concentration of acetamide increased (0-200 mmol/L), the activity of *NAGase* rapidly decreased, as shown in Fig. 1A. The IC_{50} value of acetamide was estimated to be 15 mmol/L. The inhibitory effect of acetamide tended to flatten at acetamide concentrations of 200-1200 mmol/L, as shown in Fig. 1B.

Determination of the Inhibition Mechanism and Inactivation Type of Acetamide on Boar Semen *NAGase*

The inhibition type of boar semen *NAGase* by acetamide was studied. Plots of enzyme activity versus concentration in the

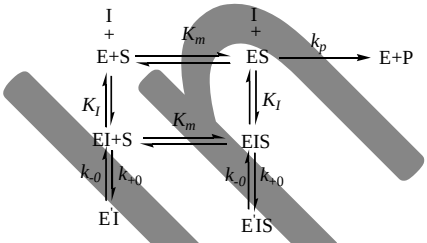
presence of different concentrations of acetamide (0, 5, 10, 15 and 20 mmol/L) produced straight lines, which all passed through the origin (Fig. 2). With the enhancement of acetamide concentration, the slope of curve increased. These changes were considered to be a reversible type of inhibition (of boar semen *NAGase* by acetamide).

The mechanism of inhibition of boar semen *NAGase* by acetamide was examined using Lineweaver-Burk plots. Increasing concentrations of acetamide produced a family of straight lines with increasing slopes and a common intercept on the *x* axis (Fig. 3A), which means V_m changed with different acetamide concentrations while K_m was unchanged. These results showed that the inhibition mechanism of acetamide on the enzyme was a non-competitive type. The inhibition constant (K_I) for acetamide was determined plotting straight-line slopes from Fig. 3A against the respective acetamide concentrations (Fig. 3B). KI was the negative value of the y axis intercepts shown in Fig. 3B, and was calculated to be 2.23× 10-2 mmol/L.

Inhibition Kinetics of Acetamide on Boar Semen *NAGase*

This study examined the kinetics of substrate reaction in the presence of different acetamide concentrations. The time course of *NAGase* catalyzing the hydrolysis of 0.2 mmol/L *pNP-NAG* in the presence of different acetamide concentrations was shown in Fig. 4A. According to Eq.4 below, the plot of ln ($[P]_{calc}$− $[P]_t$) versus time (t) generated a series of straight lines (at different concentrations of acetamide) with slopes of -*A* (Fig. 4B). The kinetic inhibition rate constant in the presence of different acetamide concentrations is shown in Fig. 4C.

According to Xie *et al.* (2006), the substrate reaction scheme can be shown on follows:



Symbols I, E, EI, S, ES, and P denote acetamide, boar semen *NAGase*, inactivated enzyme, substrate, enzyme-substrate complex, and product, respectively, andk_{+0} and k_{-0} are microscopic forward and backward rate constants of the inactivation step.

When [S] ≥ [E_0], [I] ≥ [E_0], the product formation can be shown on follows:

$$[P]_t = \frac{k_{-0}\, v}{A} \bullet t + \frac{(A - k_{-0})v}{A^2}(1 - e^{-A \bullet t}) \quad (1)$$

$$A = \frac{k_{+0}\,[I]}{K_I + [I]} + k_{-0}, \quad B = k_{-0} \quad (2)$$

Table 1: The pH value and osmotic pressure of the measurement system with different concentrations of acetamide

Acetamide concentrations (mmol/L)	0	15	50	100	200
pH value	7.34	7.3	7.45	7.65	7.78
Osmotic pressure (mOsm/kg)	320	286	367	389	421

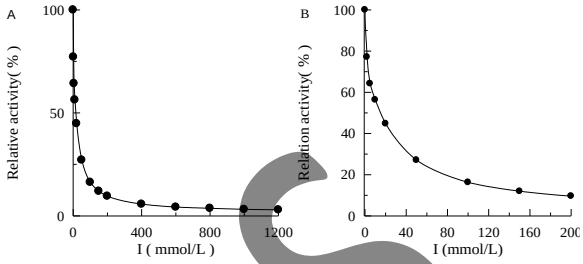


Fig. 1: Effects of acetamide concentrations on the activity of *NAGase*
(A) Acetamide concentrations were 0–1200 mmol/L
(B) Acetamide concentrations were 0–200 mmol/L

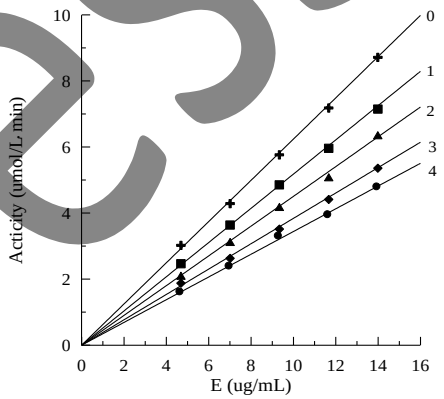


Fig. 2: Inhibition mechanism of acetamide on *NAGase*
Plots 0–4correspond to the results using acetamide concentrations of 5, 10, 15, and 20 mmol/L, respectively

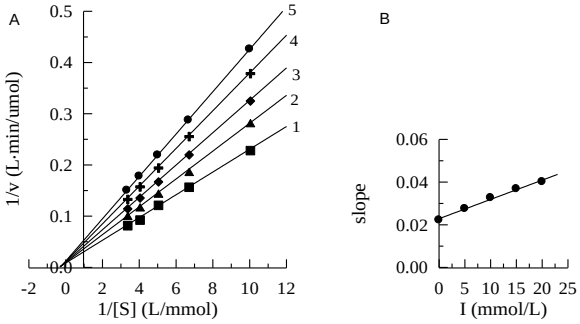


Fig. 3: Lineweaver-Burk Plots for the *NAGase* inhibition by acetamide
(A) Plots 1–5 correspond to results using acetamide concentrations of 0, 5, 10, 15, and 20 mmol/L, respectively

The $[P]_t$ denotes the concentration of product formed at time *t*, which is the reaction time. *A* and *B* are the apparent rate constants for inactivation and reactivation, respectively.

Table 2: Inhibition parameters for *NAGase* by acetamide

c/mmol L^{-1}	0.1M	0.2M	0.4M	0.6M
c-^{1}/mmol^{-1}.L	5	4	3.33	1.66
A/min^{-1}	0.02527	0.00255	0.00258	0.00265
Vk_{-0}/A^{-1}	0.00642	0.006182	0.00594	0.00571
k_{-0}/min^{-1}	0.0124	0.0121	0.0117	0.0116
A-k_{-0}	0.0126	0.0134	0. 0141	0.0149
$(A\text{-}k_{-0})^{-1}$	79.36	74.62	70.92	67.11

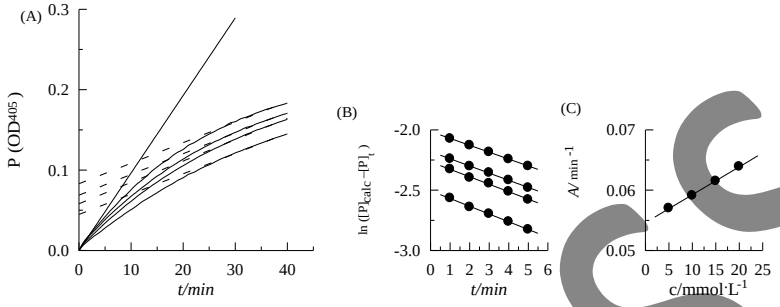


Fig. 4: Inhibition kinetics for *NAGase* by acetamide at different concentrations
(A) Curves 1–4 correspond to time courses of the substrate reaction in the presence of 5, 10, 15, and 20mmol/L acetamide, respectively
(B)Semilogarithmic plot of ln ($[P]_{calc}-[P]_t$) against time using data from curves 1–4 in (A)
(C) Apparent forward inhibition rate constants versus different acetamide concentrations

When t is sufficiently large, the curves become straight lines and the product concentration is shown on $[P]_{calc}$:

$$[P]_{calc} = \frac{k_{-0}\, v}{A} \cdot t + \frac{(A - k_{-0})\, v}{A^2} \quad (3)$$

A plot of $[P]_{calc}$ versus t gives a straight line with the slope of k_{-0}/A from Eq. 3

$$\text{slope} = \frac{k_{-0}\, v}{A} \quad (4)$$

therefore:

Combining Eq. 1 and Eq. 3 yields:

$$[P]_{calc} - [P]_t = \frac{(A - k_{-0})\, v}{A^2} \cdot e^{-A \cdot t} \quad (5)$$

$$\ln([P]_{calc} - [P]_t) = \ln \frac{(A - t_{-0})\, v}{A^2} - A \cdot t \quad (6).$$

Plotting ln ($[P]_{calc}-[P]_t$) versus initial reaction time t gives a family of straight lines at different concentrations of acetamide with slopes of $-A$. A plot of $[P]_{calc}$ versus t gives a straight line with the slope of k_{-0}/A from Eq. 3, and we calculatedk_{-0}=1.49 × 10^{-3} min^{-1}.
Combining Eq. 2 yields:

$$\frac{I}{A - k_{-0}} = \frac{K_I}{k_{+0}} \cdot \frac{1}{[I]} + \frac{1}{k_{+0}} \quad (7)$$

A plot of $1/(A-k_{-0})$ against 1/[I] gives a straight line for the slope of K_I/k_{+0}, the vertical intercept of $1/k_{+0}$, and the horizontal intercept of $-1/K_I$. Then, the microscopic rate constant, k_{+0}, and the equilibrium constant for inhibitor binding, K_I, were calculated. These rate constants are shown in Table 2. The value of the microscopic rate constant k_{-0} was similar at different acetamide concentrations.

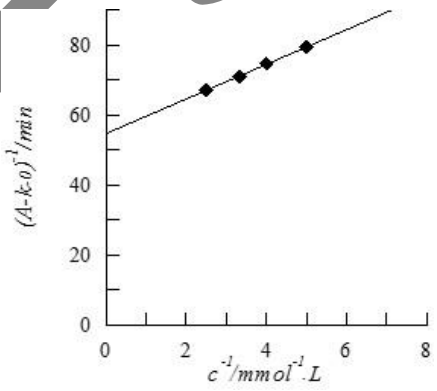


Fig. 5: Plot of $(A- k_{-0})^{-1}$ versus the reciprocal of acetamide concentrations

From Eq. 7, a plot of $1/(A- k_{-0})$ against 1/[I] gives a straight line (Fig. 5). The current results conform to the least-squares analysis. The vertical intercept and horizontal intercept represent $1/k_{+0}$ and $-1/K_I$, respectively. Therefore, we calculated the values ofk_{+0} (1.81 × 10^{-2} min^{-1}) and K_I (9.09 × 10^{-2} mmol/L).

This study found that the kinetics of substrate reaction in the presence of different acetamide concentrations. To measure the inhibition rate constant of boar semen *NAGase* by acetamide, kinetic time courses were tracked during the enzymatic hydrolysis reaction using different *pNP-NAG* concentrations with 5-mmol/L acetamide. Data plots shown that a straight line was approached at each concentration of substrate when the reaction time was sufficiently long (represented by dashed lines in Fig. 6A), and the initial reaction rate and the slope of the dashed lines increased with increasing substrate concentrations.

Table 3: Effect of acetamide on sperm motility parameters (x±s, n=3)

Time (min)	Acetamide concentrations (mmol/L)	VAP (um/s)	VCL (um/s)	VSL (um/s)	MOT (%)	PRG (%)	ALH (um)	BCF(Hz)
15	(A, group)0	55.65±2.51	74.64±4.39	49.13±1.79	89.00±5.13	45.53±5.86	3.79±0.09	28.85±0.62
	(B, group)10	41.97±0.81**	57.02±5.87**	37.89±1.14**	81.57±6.25	25.07±4.05**	3.08±0.20**	30.36±1.57
	(C, group)50	45.75±1.74**	63.94±3.06**	40.99±0.98**	88.87±6.53	31.53±2.86**	3.38±0.09**	29.08±0.92
	(D, group)100	44.53±2.02**	60.83±0.95**	40.41±2.14**	83.70±3.33	29.47±4.06**	3.07±0.02**	27.87±1.19
	(E, group)200	41.97±2.45**	58.17±2.98**	38.17±2.28**	86.10±6.35	26.27±4.85**	2.96±0.15**	28.94±0.28
30	(A, group)0	55.65±2.51	61.13±1.56	40.71±1.08	87.07±1.88	32.03±2.11	3.12±0.24	28.42±0.30
	(B, group)15	41.97±0.81**	49.68±2.37**	30.28±2.23**	83.53±3.37	15.37±1.24**	2.69±0.13*	29.54±1.28
	(C, group)50	45.75±1.74**	52.63±2.31**	34.26±2.07**	86.53±1.01	20.77±0.81**	2.49±0.28**	27.83±0.28
	(D, group)100	44.53±2.02**	50.40±3.27**	30.32±0.32**	85.53±3.94*	21.23±1.71**	2.33±0.13**	26.66±0.08*
	(E, group)200	41.97±2.45**	49.23±2.94**	32.41±1.44**	85.30±5.23	16.67±1.75**	2.27±0.13**	28.16±1.27*
60	(A, group)0	57.85±3.50	61.13±1.56	81.73±1.36	87.86±1.89	39.47±7.50	3.53±0.40	29.09±1.22
	(B, group)15	31.14±1.37**	49.68±2.37**	55.02±2.64**	79.87±2.89**	15.37±1.24**	2.27±0.24**	27.79±0.65
	(C, group)50	41.36±1.88**	52.63±2.31**	56.96±2.63**	76.36±0.90**	20.77±0.81**	2.26±0.11**	27.26±1.46
	(D, group)100	40.54±1.09**	50.40±3.27**	52.07±0.46**	70.57±2.11**	21.23±1.71**	2.49±0.19**	27.13±0.67*
	(E, group)200	41.17±4.42**	49.23±2.94**	52.05±3.66**	72.33±2.06**	16.67±1.75**	2.36±0.29**	27.25±1.02*

Note: Compared with the control group (A) *: $P<0.05$, **:$P<0.01$

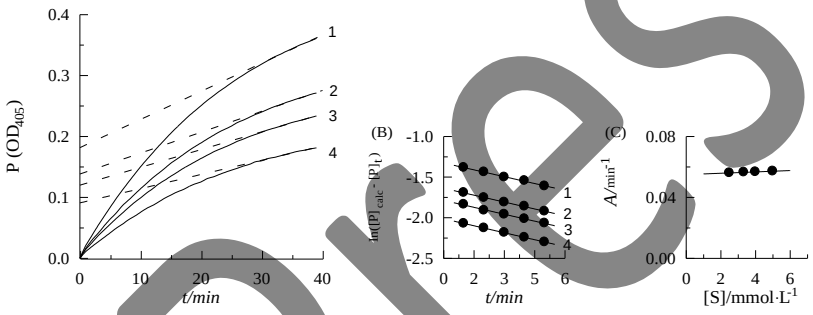


Fig. 6: Time course of the substrate reaction in the presence of 5 mol/L acetamide
(A) Progress curves 1–4 are shown for substrate concentrations of 0.20, 0.25, 0.30 and 0.40 mmol/L, respectively
(B) Semilogarithmic plot of In ($[P]_{calc}-[P]_t$) against time with data taken from curves 1−4 in (A)
(C) Plot of the apparent forward inhibition rate constants *A* versus substrate concentrations

Plots of ln ($[P]_{calc}-[P]_t$) versus *t* at the beginning of the reaction give a family of straight lines (for different acetamide concentrations) with slopes of *A* as shown in Fig. 6B. A plot of the apparent inactivation rate constant versus *pNP-NAG* concentration [S] gives a horizontal straight line (Fig. 6C). The current results indicated that the microscopic rate constants k_{-0} and k_{+0} were not a function of the *pNP-NAG* concentration.

Effect of Acetamide on Sperm Motility Parameters

Effects of different acetamide concentrations on porcine sperm motility and movement parameters were shown in Table 3. Compared with the control group, the sperm motion parameters (VAP, VCL, VSL, ALH and PRG) were decreased significantly ($P<0.01$) after incubation with 15, 50, 100 or 200 mmol/L acetamide. In addition, sperm MOT% and BCF in boar semen had decreased significantly ($P < 0.05$) after 30 min incubation with 100 mmol/L acetamide. After a 60-min incubation, sperm MOT% was significantly ($P<0.01$) lower than that for the control group, and appeared to decrease with increasing acetamide concentrations.

Discussion

The effect of an effectors on NAGase has been reported. This study found that acetamide had a reversible and non-competitive inhibitory effect on *NAGase* activity in pig spermatozoa, similar to the inhibitory effect of fructose (Farooqui and Srivastava 1980). The IC50 of acetamide and fructose to *NAGase* was 50 and 575 mmol/L, respectively. Therefore, acetamide was more potent at inhibiting *NAGase* activity in porcine semen. An inhibition kinetics model has shown that the microscopic inactivation rate constants k_{+0} and k_{-0} were 1.81×10^{-2} min^{-1} and 1.49×10^{-3} min^{-1}, respectively. These results indicated that acetamide inhibition of *NAGase* was a rapid combination of slow and inactivation processes. Using the calculated k_{+0} and k_{-0} values, *NAGase* was determined to be completely inactivated by high concentrations of acetamide, and the substrate had no protective effect on the enzyme.

Miller *et al.* (1993) used the *NAGase* inhibitor PUGNAC to treat mouse sperm in vitro and found that inhibition of *NAGase* activity reduced the identification of the sugar chain signal, and resulted in a decrease in the ability of spermatozoa to bind to an egg. Polish scientists found that

acetamide significantly reduced sperm motility parameters (VAP, VCL, VSL, MOT, and PRG) in Siberian sturgeon spermatozoa, and inhibition of both sperm and egg NAGase activity resulting in the fertilization rate decreased from 84% to 12% (Sarosiek *et al.,* 2014). In this study, the effects of acetamide on sperm motility parameters in the boar were determined by HT-CASA-II. The results have shown that sperm motility parameters (VCL, VAP, VSL, ALH and PRG) in porcine semen were significantly decreased with acetamide treatment. Sperm MOT was also significantly decreased, which indicated the acetamide can significantly inhibit sperm movement. The studies have shown that *NAGase* activity has functional relevance to sperm motility and fertilization rate.

Our current results found that acetamide inhibited the activity of *NAGase* in porcine sperm. The inhibitory kinetics of acetamide on NAGase activity in boar semen, and its inhibitory effect on motility parameters of spermatozoa will provide a basis for further study into the molecular mechanisms of *NAGase* that regulate sperm quality and fertilization rate. These results may ultimately lead to the identification of effective methods to improve the quality of porcine semen and fertilization rates, and provide new avenues for improving porcine reproductive performance.

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