Induction of acrosome reaction of spermatozoa in *Eriocheir sinensis* by low temperature

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Abstract The effects of temperatures, durations of treatment, and derivations from spermatophores or spermaries on in vitro acrosome reaction of the spermatozoa in the Chinese mitten crab *Eriocheir sinensis* were investigated. The results showed that the different temperatures resulted in extremely significant differences ($p < 0.01$) in the time of beginning acrosome reaction, the time of the maximum percentage of acrosome reaction, and the maximum percentage of acrosome reaction of the spermatozoa from spermatophores; and the low temperature (-20 , -80 °C and liquid nitrogen) induced acrosome reaction of more than 90% spermatozoa while 15 and 4 °C didn't. Similar results occur in the spermatozoa, treated with -80 °C for 15 min, from spermaries but the time of beginning acrosome reaction and the time of the maximum percentage of acrosome reaction were obviously longer than those from spermatophores. In conclusion, low temperature can induce acrosome reaction, which is a novel and efficient operating method of inducing acrosome reaction; the spermatozoa might be affected physiologically to capacitate with chilling. The study may be beneficial to new understandings of mechanism of acrosome reaction and provide the foundational material for artificial fertilization

and breeding of this crab and other commercial aquatic crustaceans.

Keywords *Eriocheir sinensis* · Spermatozoa · Acrosome reaction · Low temperature

Introduction

As a special kind of cell, live spermatozoa need to undergo an acrosome reaction (AR), a crucial physiological process for fertilization in most animals. The AR allows spermatozoa to fuse with and penetrate the ovum membrane for fertilization. Similar to most crustaceans, the AR of spermatozoa in the Chinese mitten crab *Eriocheir sinensis* (Crustacea: Decapoda) consists of four steps (Du and Xue 1987; Kang et al. 2009; Li et al. 2010): (a) the initiation of the reaction (the protrusion of the apical cap), (b) the reversion of the acrosomal vesicle, (c) the extension of the acrosomal tubule, and (d) shrinking and disappearance of the acrosomal vesicle and diminishing of nucleus. However, many factors are essential for the induction of in vivo or in vitro AR (Du and Xue 1987; Kang et al. 2009; Li et al. 2010; Lindsay and Clark 1992; Wang 1995). Temperature, which is one of the most important factors that affect the physiology of cell (Boon et al. 2008; Fan et al. 2008; Jong et al. 2009; Oguchi et al. 2006), is one of them (Martin et al. 2001; Nagao and Munehara 2007). Low temperature has been widely used for in vitro spermatozoa storage and

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artificial fertilization of aquatic crustaceans (Bart et al. 2006; Magda et al. 2004; Nimrat et al. 2006; Thomas et al. 1988; Vuthiphandchai et al. 2007). However, the information on the effects of temperature on AR and capacitation in crabs are sparse (Bhavanishankar and Subramoniam 1997; Kang et al. 2009) although aquaculture of crabs has become very important. *E. sinensis* is an indigenous and commercially important aquatic crustacean in China, but can also be found as an invasive species in Europe and America. Despite its ecological and economic importance, the relationships of temperature with in vitro AR and the morphology of spermatozoa, which may provide an indication of fertility even clues to the nature of the capacitation process, are not well understood.

The objective of the present study is to investigate the effects of five temperatures (15, 4, -20 , -80 °C, and liquid nitrogen), three durations of treatment (5, 10 and 15 min), and two derivations of spermatozoa (from spermatophores or spermaries) on in vitro AR in *E. sinensis*. Firstly, the changes in the morphology, the time of beginning AR (TBA), the time of the maximum percentage of AR (TMA), and the maximum percentage of AR (MPA) under the various conditions mentioned above were observed because they are important parameters of spermatozoal viability (Kang et al. 2009). Then, the TBA, TMA and MPA of the spermatozoa, treated with -80 °C for 15 min, from spermaries were assessed. Finally, the relationships of the changes in AR with temperatures of treatment, durations of treatment, and derivations of the spermatozoa were speculated. The present study may be beneficial to new understandings of mechanism of AR, and provide the foundational material for artificial fertilization and breeding of this crab and other commercial aquatic crustaceans.

Materials and methods

Spermatozoal collection

Adult male *E. sinensis* (150–180 g in wet weight) from Baiyangdian Lake in Anxin County, Hebei Province, China in November, 2006 were used in the experiments.

The spermatozoa were obtained from spermatophores or spermaries (Kang et al. 2009; Li et al. 2010). Firstly, the spermatophores or spermaries were placed

in precooled (4 °C) Ca^{2+} -free artificial seawater (including 370 mM/L NaCl, 15 mM/L KCl, 9 mM/L H_3BO_3 , 5 mM/L NaOH and 20 mM/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH8.2) (Goudeau and Goudeau 1986). After 10 min of settlement at 4 °C, the precipitate of the spermatophores or spermaries were resuspended with the same buffer, and then were ground and triturated with an glass homogenizer in an ice-bath. Settled for a further 10 min, the resulting supernatants were centrifuged at $220 \times g$ for 10 min at 4 °C to separate the spermatozoal suspension and the precipitable fragments of spermatophores or spermaries. The latter supernatant was then further centrifuged at $800 \times g$ for 10 min at the same temperature and the precipitate is spermatozoa, which were resuspended in Ca^{2+} -free artificial seawater to give a concentration of 5×10^6 spermatozoa/mL.

Spontaneous AR percentage and mortality of fresh spermatozoa from spermatophores

In addition, assessment of living and dead spermatozoa was accomplished by using the vital dye Eosin Y (in aqueous solution) (Kang et al. 2009) to estimate whether the sperm cells could be used as material in the experiments. The spermatozoa that stained wholly or partly red were considered to be dead ($n = 3$).

Assessment of acrosomal status of spermatozoa from spermatophores

The fresh spermatozoa obtained above were resuspended in Ca^{2+} -free artificial seawater in 1 mL aliquots in cryovials and divided into five groups, which were subsequently transferred to 15, 4, -20 , -80 °C, and liquid nitrogen (-196 °C). At 5, 10 and 15 min of treatment with the given temperature ($n = 3$), the acrosomal status of the spermatozoa was determined to assess the effects of the temperatures on AR by examining and photographing with an optical light microscope at $\times 400$ magnification.

Assessment of TBA and TMA of spermatozoa from spermatophores

About 200 random sperm cells in each sample, which was thawed in a water bath at 37 °C for 3 min, were assessed to yield the TBA and TMA. It was referred

to the method of Kang et al. (2009). The spermatozoa that were undergoing or had undergone the process of acrosomal tubule extending out of the acrosomal tubule were considered as AR, and the others as unreacted ones.

Assessment of MPA of spermatozoa from spermatophores

The MPA of each sample was also assessed. After the treatment mentioned above, about 200 random spermatozoa in each sample were estimated by photographing and examination with an optical microscope at $\times 400$ magnification to yield the percentage of AR, according to the method of Kang et al. (2009).

Analysis of data of spermatozoa from spermatophores

Data were subjected to an analysis of variance (ANOVA) with respect to the TBA, TMA and MPA. Where significant effects were found, differences between observations were quantified by calculation of the significant differences. And differences between the effects of duration, temperatures of treatment on the TBA, TMA and MPA were compared and subjected to univariate and bivariate analysis of variance (*q*-Test), if necessary.

Assessment of AR of spermatozoa from spermaries

The methods referred to those of the spermatozoa from spermatophores above. But in this section, we only assessed spontaneous AR percentage, mortality of fresh spermatozoa, acrosomal status, TBA, TMA, and MPA at -80°C for 15 min of treatment according to the data of the spermatozoa from spermatophores obtained above. Differences of the TBA, TMA and MPA between the spermatozoa from spermatophores and those from spermaries were compared and subjected to an ANOVA.

Results

Spontaneous AR percentage and mortality of fresh spermatozoa

The statistical analysis in this study shows that the spontaneous AR percentages of the fresh spermatozoa were $1.33 \pm 0.58\%$ (from spermatophores, $n = 3$) and $0.33 \pm 0.58\%$ (from spermaries, $n = 3$) and that the mortalities of the fresh-spermatozoa were $3.33 \pm 1.53\%$ (from spermatophores, $n = 3$) and $0.50 \pm 0.50\%$ (from spermaries, $n = 3$), which show that the spermatozoa are initially of good quality and can be used as material in the later experiments.

Fig. 1 Effect of temperature on acrosome reaction (AR). **a** 15 and 4°C cannot induce AR of the spermatozoa; **b–e** -20 , -80°C and liquid nitrogen can efficiently induce AR and the majority of spermatozoa undergo AR gradually from step 1 to step 4; **b** step 1 of AR (the initiation of the reaction and the protrusion of the apical cap); **c** step 2 of AR

(the reversion of the acrosomal vesicle); **d** step 3 of AR (the extension of the acrosomal tubule); **e** step 4 of AR (shrinking and disappearance of the acrosomal vesicle and diminishing of nucleus); the arrows indicate the spermatozoa at the various given steps of AR in the corresponding pictures; the scale bar = $10\text{ }\mu\text{m}$

Profile and MPA of spermatozoa from spermatophores

The acrosome-reacted spermatozoa under the various temperatures were separately profiled (Fig. 1) after about 1.5 h of post-thaw. The majority (more than 90%) of spermatozoa from spermatophores at -20 , -80 °C, and in liquid nitrogen (-196 °C) underwent AR gradually from step 1 to step 4 of AR in about 1.5 h (Fig. 1). Furthermore, the steps of AR were synchronous in most spermatozoa. In contrast, the spermatozoa at 15 and 4 °C mostly presented unchanged spherical form and a few of them underwent partial AR, only approaching the step of extending out of the acrosomal tubule, and here the MPA occurred after about 1.5 h was just only approximate 5%. Statistic analysis shows that there was a significant correlation ($p < 0.01$) between the MPA and the temperature of treatment (Fig. 2). There were extremely significant differences ($p < 0.01$) between the temperatures of treatment except for between -20 , -80 °C and liquid nitrogen, and between 15 and 4 °C ($p > 0.05$) in the MPA. Further analysis shows that there were significant differences ($0.05 > p > 0.01$), but not an extremely significant difference ($p < 0.01$), between the durations of treatment except for between 5 and 10 min ($p > 0.05$) in the MPA at -20 , -80 °C, and in liquid nitrogen. There was no significant difference ($p > 0.05$) in the MPA neither between 5 and 10 min

Fig. 2 Effect of temperature and duration of treatment on the maximum percentage of acrosome reaction. Differences of majuscules on all bars indicate extreme significance of difference ($p < 0.01$) between each other; differences of lowercases on the same temperature bars indicate significance of difference ($0.05 > p > 0.01$) between each other; same lowercases on the same temperature bars indicate no significance of difference ($p > 0.05$) in this temperature group; LN liquid nitrogen; data in figure are means $\pm$ SEM ($n = 3$)

at -20 , -80 °C, and in liquid nitrogen nor between the durations of treatment at 4 and 15 °C.

TBA and TMA of spermatozoa from spermatophores

Both the TBA (Fig. 3) and the TMA (Fig. 4) of the spermatozoa from spermatophores also varied with the different temperatures of treatment ($p < 0.01$) but not with the three different durations of treatment ($p > 0.05$). The lower the temperature was, the shorter both the TBA and the TMA were. The differences (q -Test) between the temperatures of treatment except for between 15 and 4 °C and between -80 °C and liquid nitrogen are extremely significant (with the total mean TBA being 20.78 min at 15 °C compared with 10.33 min in liquid nitrogen)

Fig. 3 Effect of temperature and duration of treatment on the time of beginning acrosome reaction. Differences of majuscules on all bars indicate extreme significance of difference ($p < 0.01$) between each other; identical lowercases on the same temperature bars indicate no significance of difference ($p > 0.05$) in this temperature group; LN liquid nitrogen; data in figure are means $\pm$ SEM ($n = 3$)

Fig. 4 Effect of temperature and duration of treatment on the time of the maximum percentage of acrosome reaction. Differences of majuscules on all bars indicate extreme significance ($p < 0.01$) of difference between each other; identical lowercases on the same temperature bars indicate no significance of difference ($p > 0.05$) in this temperature group; LN liquid nitrogen; data in figure are means $\pm$ SEM ($n = 3$)

Fig. 5 Comparison of the time of beginning acrosome reaction (TBA), the time of the maximum percentage of acrosome reaction (TMA), and the maximum percentage of acrosome reaction (MPA) of the spermatozoa from the spermaries with the spermatophores. Data in figure are means $\pm$ SEM ($n = 3$)

($p < 0.01$) (Fig. 3). A similar pattern was observed for the TMA (Fig. 4).

AR of spermatozoa from spermaries

Similar to the spermatozoa from spermatophores, the majority ($92.06 \pm 5.45\%$) of spermatozoa from spermaries, treated with -80°C for 15 min, also underwent AR gradually until step 4 of AR. But their TBA and TMA were 41.67 ± 7.64 min and 143.33 ± 25.17 min, which were obviously longer than those of the spermatozoa from spermatophores (Fig. 5). Moreover, the steps of AR were asynchronous in most spermatozoa.

Discussion

The results in this study indicate that different temperatures of treatment had different effects on AR (Figs. 1, 2, 3 and 4). The spermatozoa from the spermatophores treated with -20 , -80°C and liquid nitrogen, might undergo serial changes of acrosomal status shortly after the treatment and then accomplished AR in about 1.5 h (Fig. 1). The treatment with -20 , -80°C , and liquid nitrogen also resulted in significant decreases in both the TBA and the TMA and a remarkable increase in the MPA (Figs. 2, 3 and 4). However, the treatment of 15 and 4°C could not induce AR. The results showed that the TBA, TMA and MPA were remarkably correlative to the temperatures of treatment, and that the differences

between the various temperatures (except for between -80°C and liquid nitrogen, and between 15 and 4°C) both in the TBA and in the TMA were extremely significant ($p < 0.01$). However, no similar statistically significant differences were found between the different durations of treatment, or any combination of variables. In addition, the effect of the treatment of -20°C on AR was slighter than those of -80°C and liquid nitrogen.

The results elucidate that the treatment of spermatozoa with low temperature (-20 , -80°C and liquid nitrogen) is an efficient and operating method of induction of AR. To date, only A23187 (Medina and Rodriguea 1992) or egg-water (Du and Xue 1987; Wang 1995) are widely used for the induction of AR in Decapoda. However, the effect of egg-water on induction of AR is fluctuant and ambiguous due to the difference of ova maturity (Wang 1995) and A23187 is not inexpensive. Our results indicate that the treatment of low temperature is an inexpensive method possessing high utility and stable effect of induction of AR.

The differences of both the TBA and the TMA, and the similarity of MPA indicate that the spermatozoa from the spermatophores and the spermaries possess different levels of maturity and the MPA do not correlate to the derivations of spermatozoa. High level of maturity of the spermatozoa from spermatophores caused short TBA and TMA while low level of maturity of the spermatozoa from spermaries caused long ones however their final MPA were similar (Fig. 5). The result also remarkably demonstrates that before AR *E. sinensis* spermatozoa possess the physiological process relating to AR. It might be the process of capacitation, which has been found in the shrimp *Sicyonia ingentis* (Crustacea: Decapoda) (Lindsay and Clark 1992) and *Litopenaeus* (Crustacea: Decapoda) (Alfaro et al. 2007) but has not been known in *E. sinensis*. But our result, that the spermatozoa treated by low temperature but not by 15 and 4°C can undergo AR, indicates that the spermatozoa can be affected physiologically to capacitate with low temperature, which is coincident with the results published by Du and Xue (1987). Capacitation is the essentially physiological process prior to AR in most animals and the AR might be initiated only if this process occurs. Therefore, capacitation sometimes is considered as the initial process of AR and capacitated spermatozoa can

promptly undergo AR. In our study, it is the low temperature that initiates the capacitation and the following AR.

Some suggest that AR results from increased intracellular calcium because calcium ionophore A23187 induces AR in media containing calcium (Medina and Rodriguea 1992). However our results indicate that the AR can be successfully induced in vitro with low temperature in media without calcium, which has been reported by Kang et al. (2009). It is possible that the proteins inhibiting AR were destructed or the proteins promoting AR were activated by low temperature, which differs from other reports (Du and Xue 1987; Li et al. 2010; Medina and Rodriguea 1992).

These acrosome reacted spermatozoa also have significant roles in scientific research. In other species, there is increasing interest in methods to produce acrosome reacted spermatozoa for injection into ova because the removal of the acrosome or at least its disruption may improve the success of the intracytoplasmic spermatozoa injection procedure (Takeuchi et al. 2004). In addition, it may be easier to extract nuclear proteins from acrosome reacted spermatozoa than from unreacted ones for further studies (unpublished).

In conclusion, low temperature (−20, −80 °C and liquid nitrogen) can effectively alter AR of *E. sinensis* spermatozoa while 15 and 4 °C cannot. Low temperature treatment is a novel, efficient, and operating method of inducing AR, even in media without calcium. After low temperature treatment, the spermatozoa might be affected physiologically to capacitate. Further studies will be necessary to assess how in vitro induction of AR by low temperature impacts the fertility of *E. sinensis* spermatozoa.

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References

- Alfaro J, Ulate K, Vargas M (2007) Sperm maturation and capacitation in the open thelycum shrimp *Litopenaeus* (Crustacea: Decapoda: Penaeoidea). *Aquaculture* 270: 436–442
- Bart AN, Choosuk S, Thakur DP (2006) Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). *Aquac Res* 37: 523–528
- Bhavanishankar S, Subramoniam T (1997) Cryopreservation of spermatozoa of the edible mud crab *Scylla serrata* (Forsk.) *J Exp Zool* 277:326–336
- Boon CH, Catherine MC, Shubhayu B (2008) Temperature and calcium ions affect aggregation of mesenchymal stem cells in phosphate buffered saline. *Cytotechnology* 58: 69–75
- Du N, Xue L (1987) Induction of acrosome reaction of spermatozoa in the decapoda *Eriocheir sinensis*. *Chin J Oceanol Limnol* 5:118–123
- Fan Y, He C, Liu G, Zhang H (2008) Optimization of the isolation and cultivation of Cyprinus carpio primary hepatocytes. *Cytotechnology* 58:85–92
- Goudeau M, Goudeau H (1986) The resumption of meiotic maturation of the ovum of the prawn *Palaemon serratus* is regulated by an increase in extracellular Mg^{2+} during spawning. *Dev Biol* 118:361–370
- Jong HN, Myriam E, Susan TS (2009) The effects of micro-carrier culture on recombinant CHO cells under biphasic hypothermic culture conditions. *Cytotechnology* 59:81–91
- Kang X, Li G, Mu S, Guo M, GE S (2009) Acrosome reaction of Chinese mitten-handed crab *Eriocheir sinensis* (Crustacea: Decapoda) spermatozoa: promoted by long-term cryopreservation. *Aquaculture* 295:195–199
- Li G, Kang X, Li Y, Mu S, Guo M (2010) Acrosome reaction of spermatozoa in the Chinese mitten crab, *Eriocheir sinensis* (Decapoda, Grapsidae): induced by anti-spermatozoal membrane proteins antiserum. *Crustaceana* (in press)
- Lindsay LL, Clark WH Jr (1992) Preloading of micromolar intracellular Ca^{2+} during capacitation of *Sicyonia ingentis* sperm, and the role of the pHi decrease during the acrosome reaction. *J Exp Zool* 262:219–229
- Magda L, Clarissa G, Marcela S (2004) The use of flow cytometry in the evaluation of cell viability of cryopreserved sperm of the marine shrimp (*Litopenaeus vannamei*). *Cryobiology* 48:349–356
- Martin P-V, William AB, Addison LL, Delbert MG, Mayra LG-F (2001) Effect of temperature on sperm quality of captive *Litopenaeus vannamei* broodstock. *Aquaculture* 198:209–218
- Medina A, Rodriguea A (1992) Structural changes in sperm from the fiddler crab, *Uca tangeri* (Crustacea, Brachyura), during the acrosome reaction. *Mol Reprod Dev* 33:195–201
- Nagao J, Munehara H (2007) Characteristics of broods fertilized with fresh or stored sperm in the helmet crab *Telmessus cheiragonus*. *J Crustacean Biol* 27:565–569
- Nimrat S, Siriboonlamom S, Zhang S, Xu Y, Vuthiphanchai V (2006) Chilled storage of white shrimp (*Litopenaeus vannamei*) spermatophores. *Aquaculture* 261:944–951
- Oguchi S, Hiroyuki S, Masayoshi T, Haruhiko T (2006) pH Condition in temperature shift cultivation enhances cell longevity and specific hMab productivity in CHO culture. *Cytotechnology* 52:199–207
- Takeuchi T, Colombero LT, Neri QV, Rosenwaks Z, Palermo GD (2004) Does ICSI require acrosomal disruption? An ultrastructural study. *Hum Reprod* 19:114–117

- Thomas A, Crowe JH, Griffin FJ, Clark WH Jr (1988) Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. Cryobiology 25:238–243
- Vuthiphandchai V, Nimrat S, Kotcharat S, Bart AN (2007) Development of a cryopreservation protocol for long-term storage of black tiger shrimp (*Penaeus monodon*) spermatophores. Theriogenology 68:1192–1199
- Wang Q (1995) Egg water induced reaction and biostain assay of sperm from marine shrimp *Penaeus vannamei*: dietary effects on sperm quality. J World Aquac Soc 26:261–271