

RNase 9 Protein Inhibited Capacitation and Acrosome Reaction of Sperms

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Abstract

Ribonuclease 9 is a member of ribonuclease A superfamily. It is expressed only in the epididymis. However it lacks ribonucleolytic activity and its function (s) is unknown. Immunofluorescence experiments showed that the location of RNase9 protein on sperm surfaces changed during capacitation and acrosome reaction and the protein migrated from neck to acrosome cap. CTC staining was used to assess the role of RNase 9 on capacitation and acrosome reaction. Compared with the control (PBS with 3%BSA), the spontaneous acrosome reaction induced by progesterone could be suppressed by RNase 9 protein and the AR rate is $6.5 \pm 1.2\%$. The exposure of spermatozoa to RNase9 protein also produced a significant decrease in the intracellular cAMP levels during acrosome reaction with respect to control (111 ± 24.5 versus 187 ± 18 fmol/106 spermatozoa; $P < 0.05$). These experiments confirmed that RNase 9 protein significantly impaired the sperm functions, including capacitation and acrosome reaction, and provide the proof for its potential in male reproductive toxicity.

Keywords: RNase 9; Capacitation; Acrosome reaction

Introduction

Human RNase 9 protein belongs to the RNase A family member [1-3]. RNase 9 protein was mainly expressed in the endothelial cells of epididymidis and localized on the posterior equatorial region of sperms. Previous studies showed that recombinant human RNase 9 did not exhibit detectable ribonucleolytic activity against yeast tRNA, but exhibited antibacterial activity, in a concentration/time dependent manner, against *E. coli* [4]. The study of the protein in sperm maturation, motility and fertilization has yet not been reported.

Sperm capacitation is a physiological stage in which all mammalian spermatozoa must undergo before fertilization [5]. A major difficulty in the study of sperm capacitation is the absence of morphological changes during sperm capacitation.

Biochemical changes involve the removal or redistribution of epididymal proteins and seminal plasma proteins on the surface of spermatozoa, changes in membrane lipid composition, membrane protein migration, and receptor exposure [6-8]. Recently, Yanagimachi [9] presented a work hypothesis: He believed that in the course of sperm capacitation, the changes of sperm surface protein including the loss or activation of receptors, position changes et al stimulating G2 protein, activating the Ca^{2+} channel and the influx of calcium ions is the key to the activation of ultra-activation. A dependent protein kinase pathway and tyrosine kinase pathway is the main way of sperm capacitation process [10-12]. RNase 9 protein is localized on the sperm surface and whether it is related to sperm capacitation, acrosome reaction and fertilization needs further study.

Materials and Methods

Origin and treatment of semen samples

According to the World Health Organization (WHO)-recommended procedure (WHO, 1999), semen samples were collected by masturbation from healthy normozoospermic donors. All people were recruited under informed consent. Before processing, all samples were produced into disposable containers and left for at least 30 min to liquefy. The spermatozoa were washed thrice (700 g for 7 min) in BWW medium. The density of the resuspended sperm is 1.0×10^6 /ml. Smears were taken at capacitation 0 h, 3 h and 5 h respectively. After capacitation, progesterone was added to the sperms and incubated for 30 minutes at 37°C.

Chemicals and instruments

Recombinant human RNase 9 antibody was constructed by our lab. BWW capacitation medium, progesterone, chlortetracycline and propidium iodide (PI) come from Sigma Company. Goat anti-mouse IgG labeled FITC was purchased from Beijing Jerser Jinqiao Biological Technology Co. Ltd. Progesterone was freshly prepared as stock solutions in dimethyl sulfoxide (DMSO) before use. 1.5 mg chlortetracycline was dissolved in 10 ml cold TN solution and was adjusted to PH 7.8 by NaOH. Fluorescence microscopy and confocal microscopy were purchased from the German Leica.

Sperm preparation

The semen was liquefied at 37°C and separated by 45% to 90% Percoll, centrifuged 18 min at 600 g. Sperms were washed 3 time, then resuspended with Bww solution. Sperm was adjusted to the concentration of 1×10^6 /ML, incubated at 37°C 5% CO₂ incubator.

Sperm immunofluorescence

Semen samples were treated as before. Sperms were incubated in BWW for 0 h, 3 h, 5 h at 37°C 5% CO₂ incubator then induced acrosome reaction. 10 µl sperm suspension smeared and fixed for 10 min by methanol. Mouse polyclonal antibody against RNase 9 was dropped (1:400 dilution) and the smears were incubated overnight at 4°C. Sperm nuclei was stained with PI and observed by confocal laser scanning microscope. The negative control was incubated in PBS with 3% BSA (FIG. 1).

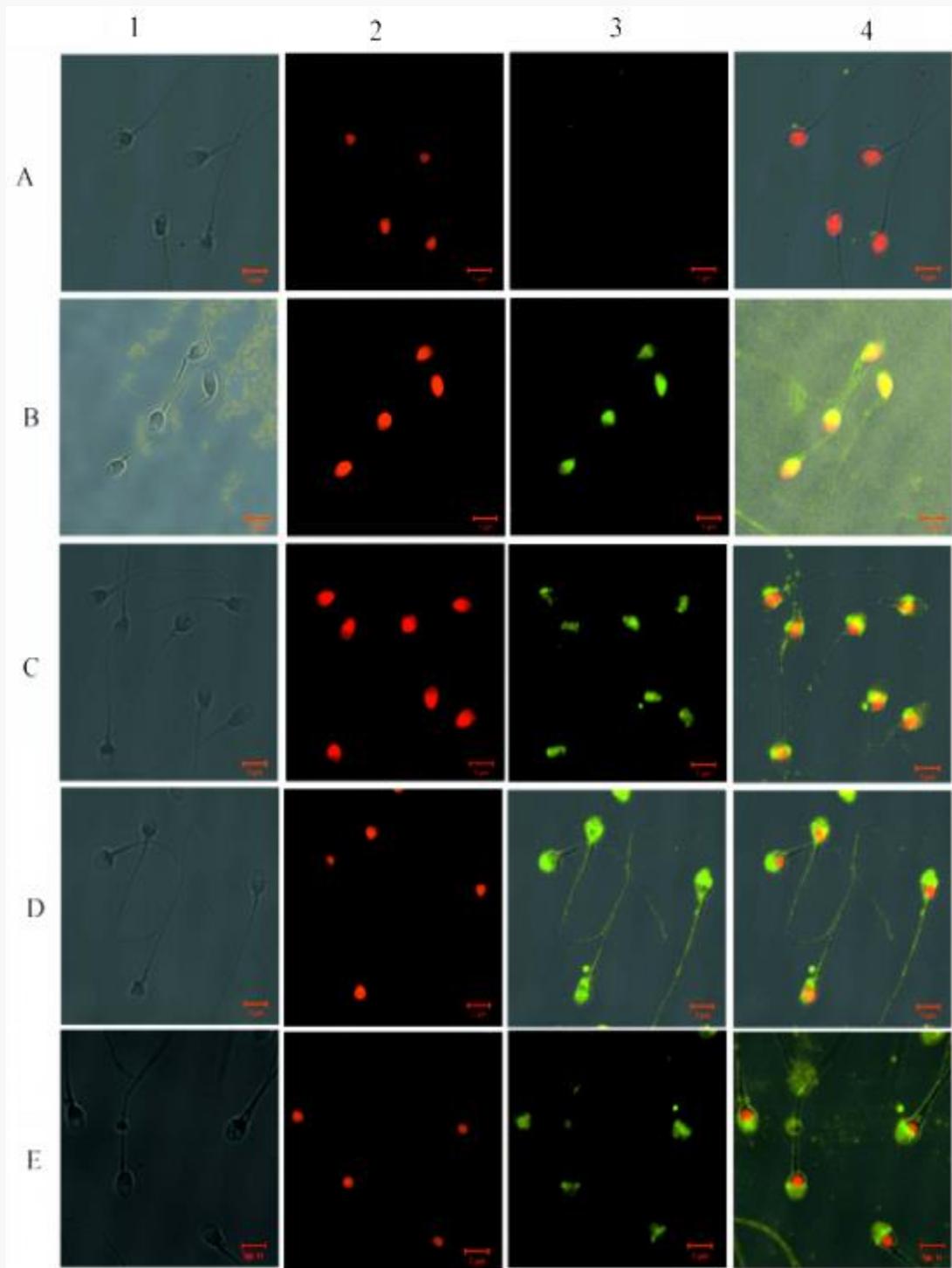


FIG. 1. Immunofluorescent staining of RNase 9 protein (A: Negative control (PBS) B: Pre-capacitation; C: Capacitation for 3 hours; D: Capacitation for 5 hours; E: Acrosome reaction. A1-E1: Black and white images of sperms; A2-E2: Nuclear staining of sperms (red fluorescent); A3-E3: Immunofluorescent staining of RNase 9 protein (green fluorescent); A4-E4: Merged images. Green fluorescent represents the location of RNase9 protein. Red fluorescent represents the location of nucleus).

Evaluation of sperm capacitation and AR

The swimming-up sperm suspensions were divided into three aliquots containing at least 10×10^6 spermatozoa. These sperms were co-cultured with RNase 9 (0.8 ug/ml), anti-RNase 9 and PBS (3%BSA) in BWW medium respectively. After 5 h incubation, we performed chlortetracycline (CTC) staining according to the procedures previously described, three types of CTC fluorescent staining (F, B and AR) could be divided: F pattern is uncapacitated spermatozoa, the acrosomal cap is intact with a strong yellow green fluorescence; B pattern is capacitated sperms. The acrosomal cap is completed with only fluorescent band in the posterior region of the acrosome cap; AR pattern displayed light green color or no fluorescence at acrosome region. At least 200 spermatozoa were counted in each smear. Three independent experiments performed in duplicate and calculate the percentage of F, B and AR pattern (FIG. 2).

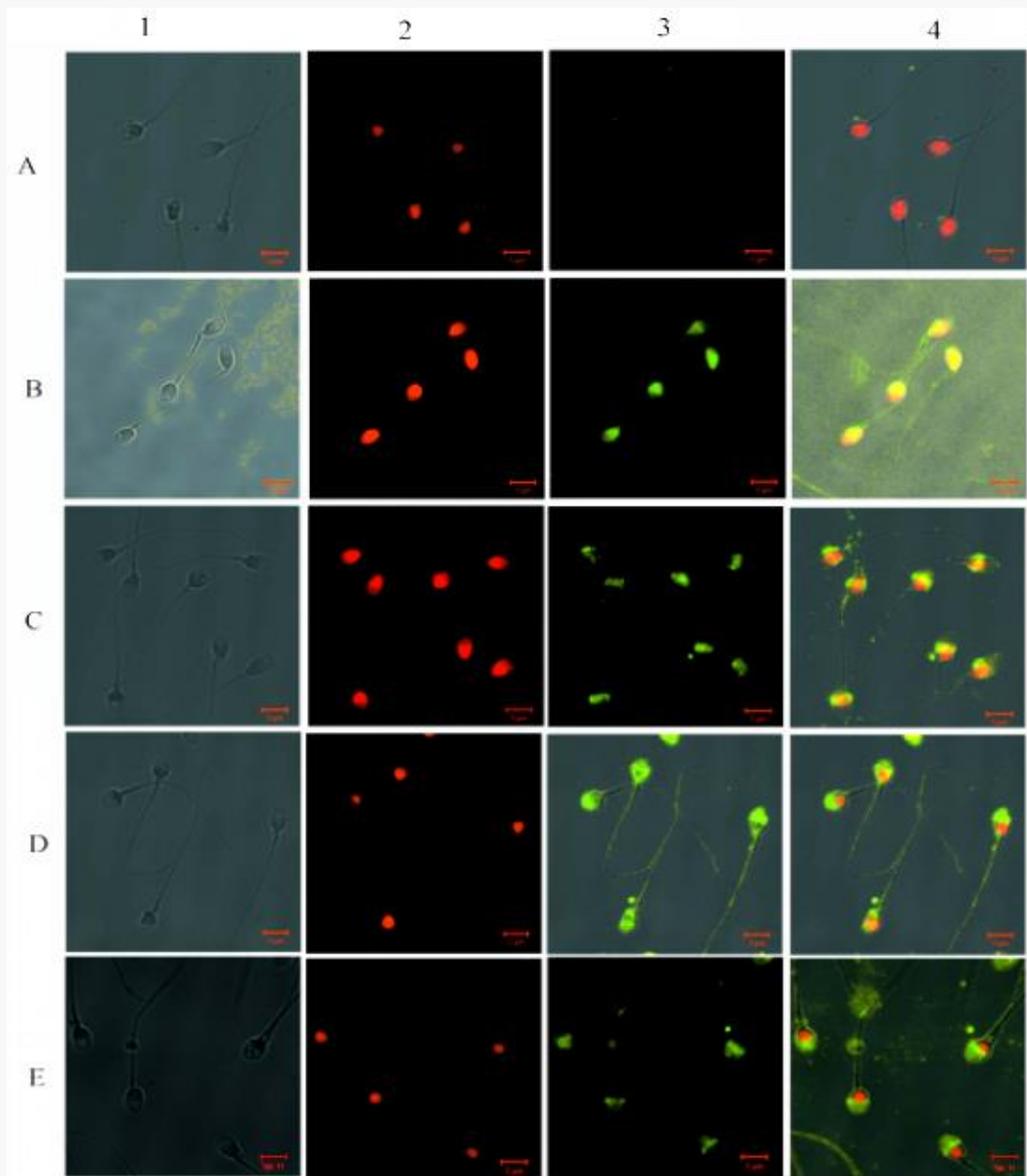


FIG. 2. Capacitation and acrosome reaction.

Cyclic AMP measurement

The swimming-up sperm suspensions were divided into three aliquots containing at least 10×10^6 spermatozoa. These sperms were co-cultured with RNase 9 (0.8 $\mu\text{g}/\text{ml}$), anti-RNase 9 and PBS (3%BSA) in BWW medium respectively. After 5 h incubation, spermatozoa were separated and the pellets were homogenized in 0.1 M HCl. The homogenates were centrifuged at 10000 g at room temperature for 30 min, and the supernatants were acetylated before determining the cAMP concentrations according to the instructions of the commercial kit manufacturer (Cyclic AMP Complete ELISA Kit, Abcam). cAMP levels were calculated in femtomole of cAMP per 10^6 spermatozoa.

The graph represents mean+SEM of three independent experiments performed in duplicate. PBS (3%BSA): Control group. * $p < 0.05$.

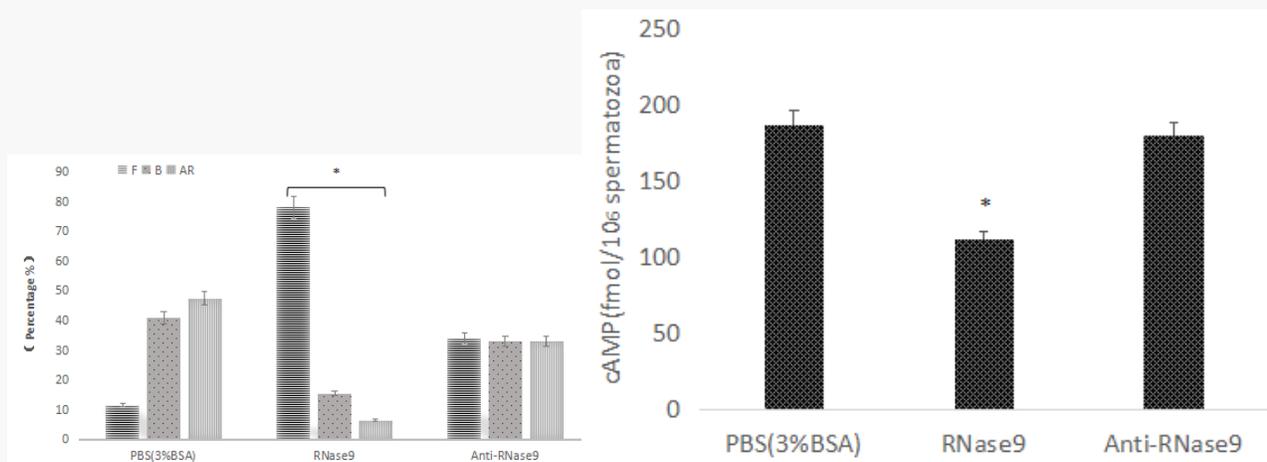


FIG. 3. cAMP levels during acrosome reaction.

Results

The changes of RNase9 localization during capacitation and AR

RNase 9 protein is localized on the head-neck region of normal sperms. During the course of capacitation, RNase 9 protein migrated from the head-neck to the equatorial region on sperms. The green fluorescence staining gradually weakened on the head-neck region of sperms for 3 hours of capacitation. For 5 hours of capacitation, there is an obvious visible green fluorescence bands at the equatorial region. Then RNase9 protein further migrated from the equatorial region to the acrosome and covering the whole acrosomal cap. During the course of progesterone induced-acrosome reaction, the color of sperm acrosome is shallow and the acrosin may be released. There is only little green fluorescence on the equatorial region.

Effect of RNase 9 on capacitation and AR

Immunofluorescence experiments found that in the process of capacitation and acrosome reaction, the localization of the RNase9 protein on the sperm surface redistributed. Whether RNase 9 protein involved in capacitation and acrosome reaction need further study. A significantly lower incidence rate of spontaneous AR ($6.5 \pm 1.2\%$; $P < 0.05$) was observed when sperms were co-cultured with RNase9 protein (0.8 ng/ml) in BWW and the F pattern rate is $78 \pm 5.6\%$ (FIG. 2). Anti-RNase9 does not affect sperm capacitation and acrosome reaction compared with PBS (3%BSA) group.

The level of intracellular cAMP

Capacitation of sperm in the female reproductive tract is the key prerequisite for the acquisition of the ability of sperm to fertilize eggs. cAMP also plays pivotal roles in molecular events leading to mammalian capacitation (De Jonge) and AR (Doherty et al.). This prompted us to measure cAMP in spermatozoa capacitated or acrosome reacted in the presence of RNase9 to analyse the potential role of this protein. As evaluated in three independent duplicate experiments, the exposure of spermatozoa to RNase9 protein (0.8 ng/ml) produced a significant decrease in the intracellular cAMP levels during acrosome reaction with respect to control (111 ± 24.5 versus 187 ± 18 fmol/106 spermatozoa; $P < 0.05$, FIG. 3). The treatment of spermatozoa with anti-RNase9 produced only a slight or no significant changes in intracellular cAMP levels with respect to control samples (179.6 ± 13.4 and 187 ± 18 fmol/106 spermatozoa ($P > 0.05$) (FIG. 3).

Discussion and Conclusion

13 members of RNase A superfamily have been found in humans. RNase9-13 is newly discovered members in male reproductive organs [13-15]. The specific expression of RNase9 and RNase10 in the epididymis of mice and pigs has been reported, but their function is unknown [16]. Mammalian sperm in the female reproductive tract can obtain the fertilization capability only undergone the physiological and morphological changes. Sperm capacitation is a multistep process and is an important physiological prerequisite for acrosome reaction, the hyper activation movement and fertilization [17]. Capacitation involves changes in sperm membrane proteins and changes in membrane fluidity until the acrosome reaction occurs. The molecular mechanism of sperm capacitation is quite complex and is not yet fully defined. The study found that sperm during capacitation, subcellular localization of RNase 9 on sperm surface changes or redistribution, suggesting that RNase9 protein may be involved in the capacitation process. CTC staining results also found that RNase9 protein inhibited sperm capacitation and acrosome reaction. Consistent with the result of CTC staining, the levels of cAMP in sperm capacitation was inhibited in the presence of RNase 9 protein. These experiments confirmed that RNase 9 protein significantly impaired the sperm functions, including capacitation and acrosome reaction, and provide the proof for its potential in male reproductive toxicity.

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