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·Original Article·

Influence of enterococci on human sperm membrane *in vitro*

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Abstract

Aim: To study the influence of enterococci on human sperm membrane *in vitro*. **Methods:** Ejaculated human sperm were artificially infected with β-hemolytic or non-β-hemolytic enterococci at the bacteria: sperm ratio of 50:1 at 37ºC. Sperm membrane integrity was examined after incubation for 1, 3 and 5 h by hypoosmotic swelling (HOS) test and electron microscopy. **Results:** Sperm infected with β-hemolytic enterococci had lower HOS scores compared with non-β-hemolytic strains or uninfected control ($P < 0.01$). The HOS test scores of sperm infected with β-hemolytic enterococci increased in the presence of phosphatidylcholine, an inhibitor of hemolysin. Non-β-hemolytic strains showed no significant difference in swelling rate, compared with the control group ($P > 0.05$). It was shown by electron microscopy that β-hemolytic enterococci caused significant rupture of human sperm membrane. **Conclusion:** β-hemolytic enterococci caused human sperm membrane injury, and might be mediated by the hemolysin of enterococci. (*Asian J Androl 2007 Jan; 9: 77–81*)

Keywords: enterococci; sperm; sperm membrane; hypoosmotic swelling test; ultrastructure

1 Introduction

Urogenital infections are important causal factors in male infertility. The most widely studied genital microorganism in relation to male infertility are gram-negative *Escherichia coli*, which are also the principal microorganism that cause prostatitis and epididymitis [1–2]. However, little is known about the influence of gram-positive uropathogenic bacteria on sperm. Enterococci are increasing causes of nosocomial infection. Besides urogenital tract infections, they also caused asymptomatic bacteriospermia and infectious epididymitis. Mehta *et al.* [3] reported that enterococcus was isolated from 53% of infected samples. In their study, the sperm concentration, as well as the percentage of morphologically normal spermatozoa, were significantly lower ($P < 0.05$) and the incidence of oligozoospermia and teratozoosper-mia was significantly ($P < 0.05$) higher in semen infected with *Streptococcus faecalis*, compared with those containing micrococci or α-haemolytic streptococci and the uninfected samples. Jacques *et al.* [4] observed no significant depressor effect of enterococci on sperm motility. Huwe *et al.* [5] reported that enterococci strains have no signifi-

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cant influence on sperm motility parameters. Hemolysin is a well-known virulence factor of enterococci, which is a pore-forming membrane toxin capable of injuring human, horse, rabbit and mouse erythrocytes, but not cow and sheep erythrocytes [6]. Can hemolysin injure human sperm? In the present study, we use β-hemolytic or non-β-hemolytic enterococci to investigate the influence of enterococci hemolysin on human sperm membrane using the hypoosmotic swelling (HOS) test and electron microscopy. To gain further insight into the possible role of hemolysin on sperm damage, phosphatidylcholine, an inhibitor of enterococci hemolysin, is used to examine whether hemolysin-induced sperm damage is blocked by phosphatidylcholine.

2 Materials and methods

2.1 Bacterial strains

Enterococci strains were obtained from clinical isolates and healthy human fecal specimens. Production of hemolysin was initially determined by plating enterococci onto brain heart infusion agar (DIFCO) supplemented with 5% rabbit blood. Plates were incubated at 37ºC and observed after 24 h. Enterococci that exhibited clear zones were considered as β-hemolytic strains. Enterococci were cultured in broth at 37ºC overnight. The suspension was adjusted to concentration of 5×10^8 cfu/mL for use.

2.2 Sperm preparation

Native ejaculates from healthy fertile donors were obtained by masturbation into sterile plastic containers. Only semen samples whose parameters corresponded with the World Health Organization norm were taken [7]. An extract of purified, highly motile spermatozoa was obtained by "swim-up" technique as described by Diemer *et al.* [2]. Sperm suspensions were diluted to concentration of 10×10^6 sperm/mL for use.

2.3 Experiment group design

The prepared sperm suspension was split into three fractions. Two samples were incubated with β-hemolytic (Group A, n = 9) or non-β-hemolytic (Group B, n = 9) enterococci at the bacteria:sperm ratio of 1:1, 10:1 or 50:1 at 37ºC for 1–5 h. The third sample was served with the culture medium as the control (n = 6).

2.4 HOS test

The HOS test was performed according to the method described by Jeyendran *et al.* [8]. Briefly, the infected sperm were centrifugated and washed, and fructose-sodium citrate hypoosmotic solution was added at a ratio of 9:1 volume, and incubated in water at 37ºC for 30 min. The HOS scores were evaluated by the other two examiners without knowledge of the experiment group division. For each β-hemolytic or non-β-hemolytic enterococci, at least 100 spermatozoa were observed in more than five random fields under a phase contrast microscope at × 400 magnification. The percentage of HOS-reacted sperm with swollen and curled tail was assessed.

For phosphatidylcholine experiment, phosphatidylcholine at concentration of 50–200 µg/mL were added to β-hemolytic enterococci suspension for 30 min, and the mixture was then incubated with sperm for 3 h.

2.5 Electron microscopy

Sperm were incubated with β-hemolytic or non-β-hemolytic enterococci at the bacteria: sperm ratio of 50:1 at 37ºC for 3 h. The infected sperm were centrifuged at $500 \times g$ for 10 min, fixed for 2 h in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4). After washing in buffer, the sperm were postfixed in 2% osmium tetroxide plus 1% potassium ferricyanide in cacodylate buffer, dehydrated through a graded alcohol series, embedded in Epon, sectioned thin, stained with uranyl acetate and lead citrate, and observed by electron microscopy.

2.6 Statistics analysis

Results of research were assessed by one-way ANOVA using SPSS11.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. $P < 0.05$ was considered significant differences.

3 Results

3.1 Effects of β-hemolytic or non-β-hemolytic enterococci on sperm membrane by HOS test

In a preliminary study, the enterococci destructed the sperm in a dose-dependent manner (data not shown). A concentration ratio of bacteria:sperm of 50:1 was found effective in a limited time course and adopted throughout the *in vitro* experiments.

There was no significant difference in swelling rate when sperm suspension was incubated with enterococci at the bacteria : sperm ratio of 50:1 for only 1 h ($P > 0.05$, Table 1). When sperm were cultured with β-hemolytic or non-β-hemolytic enterococci at the bacteria:sperm

Table 1. Swelling rate of sperm infected with β-hemolytic or non-β-hemolytic enterococci at different incubation time. Sperm were incubated with enterococci at the bacteria:sperm ratio of 50:1; data were expressed as mean ± SD; [a]$P > 0.05$, [c]$P < 0.01$, compared with group C. Group A: β-hemolytic enterococci suspension; Group B: non-β-hemolytic enterococci suspension; Group C: culture medium as control.

Incubation time (h)	Group A (n = 9)	Group B (n = 9)	Group C (n = 6)
1	80.25 ± 6.32[a]	77.26 ± 6.35[a]	79.75 ± 5.38
3	45.89 ± 5.64[c]	78.55 ± 4.26[a]	78.75 ± 5.56
5	30.67 ± 3.69[c]	79.86 ± 6.69[a]	81.32 ± 6.02

Table 2. The swelling rate of sperm infected with β-hemolytic enterococci in the absence or presence of phosphatidylcholine. Sperm were infected with β-hemolytic enterococci for 3 h in the absence or presence of phosphatidylcholine at concentrations of 50–200 µg/mL. Data were expressed as mean ± SD. [a]$P > 0.05$, [c]$P < 0.01$, compared with the absence of phosphatidylcholine.

Phosphatidylcholine (µg/mL)	Swelling sperm (%)
0	44.50 ± 4.51
50	43.00 ± 4.83[a]
100	60.25 ± 2.22[c]
200	78.75 ± 8.18[c]

ratio of 50:1 for 3 h, the incidence of swelling sperm in Group A (β-hemolytic strains) was found to be significantly lower than that in Group B (non-β-hemolytic strains), as well as that in Group C (culture medium) and the rate was even lower after 5 h ($P < 0.01$; Table 1). Non-β-hemolytic strains (Group B) showed no significant difference in swelling rate, compared with the control group ($P > 0.05$; Table 1).

3.2 Effects of phosphatidylcholine on the HOS test of β-hemolytic strains

The number of swelling sperm was higher in the presence of phosphatidylcholine than that in the absence of phosphatidylcholine. Phosphatidylcholine inhibited the hemolysin-induced membrane defect in a dose-dependent manner. When 200 µg/mL of phosphatidylcholine was used, the membrane injury was completely inhibited; the HOS test scores were not significantly different from that of non-infected control group (Table 2).

3.3 Effects of β-hemolytic or non-β-hemolytic enterococci on sperm membrane ultrastructure

To further examine the membrane impairment, sperm ultrastructure was observed by electron microscopy. The

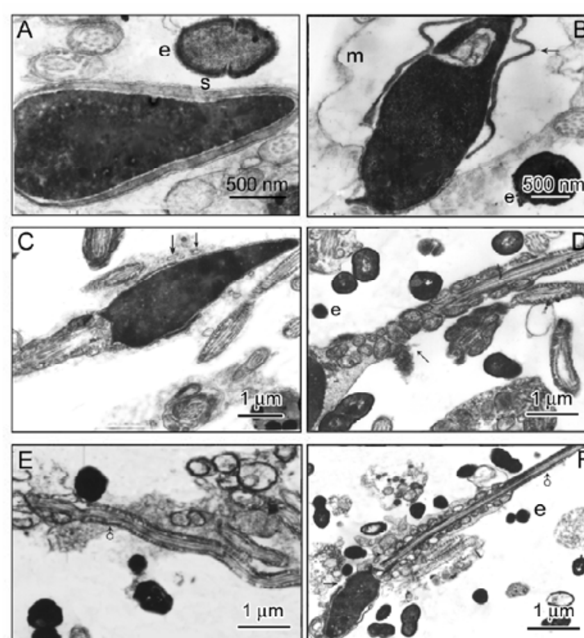

Figure 1. Ultrastructure of sperm infected with β-hemolytic or non-β-hemolytic strains. Sperm were incubated with β-hemolytic or non-β-hemolytic enterococci at bacteria: sperm ratio of 50:1 for 3 h. (e, enterococci; m, cytoplasm membrane; s, sperm;). (A): The membrane of sperm infected with non-β-hemolytic strain was almost intact. There was no defect on the membrane of sperm (× 32 000). (B): The head membrane of the sperm infected with β-hemolytic strain was swollen, deformed and obscured; the acrosome membrane was seen curled and distorted (↑) (× 21 600). (C): The head membrane of sperm infected with β-hemolytic strain had shed, only left some acrosome membrane (↑) (× 12 000). (D): The tail membrane in neck and the middle piece of the sperm infected with β-hemolytic strain was damaged; the mitochondria were disorderly arranged; some components were released from the cytoplasm (↑) (× 14 400). (E): The sperm infected with β-hemolytic strain had a comparatively intact membrane in the principal piece of the tail (♂) (× 14 400). (F) The membrane of the sperm infected with β-hemolytic strain was damaged on the head, neck, and the middle piece of the tail (↑), but the membrane in the principal and end pieces of the tail were less damaged (♂) (× 6 800).

majority of the sperm treated with non-β-hemolytic strains had a normal appearance and its membrane was practically intact (Figure 1A). However, for sperm treated with β-hemolytic strains, the membranes of their heads were swollen, deformed, obscured and even broken off. The acrosome membrane and nuclear membrane could be seen injured too, which was curled, distorted and broken off (Figure 1B, C). The membrane in the neck and the middle piece of the tail was defective. Mitochondria were disorderly arranged, and some components were released from the cytoplasm, but the membrane in the principal and the end piece of the tail were less damaged and its membranes were comparatively intact (Figure 1D–F). This indicates that hemolytic bacterium impaired significantly spermatozoa membrane.

4 Discussion

The significance of bacteriospermia for male subfertility has gained increasing attention in recent 10 years. In an analysis of clinical samples in China, enteroccocci were the second most common pathogen causing procreation system of infection [9–10]. Enterococci are important bacterium causing asymptomatic bacteriospermia and infectious epididymitis. The influence of enterococci on sperm is under debate [3–5]. Hemolysin is a pore-forming membrane toxin, which is a virulence factor of enterococci [11]. The effect of enterococci hemolysin on human sperm membrane integrity remains obscure. The functional and structural integrity of sperm membrane are crucial for the viability of spermatozoa. Based on water transport across the sperm tail membrane under hypoosmotic conditions, the HOS test has been used to assess semen quality, to analyze fertilizing capacity and also to detect viable, immotile cells for intracytoplasmic sperm injection in humans [12]. Therefore, HOS testing enables the evaluation of the functional status of the sperm membrane. Our results show that sperm infected with β-hemolytic enterococci had lower HOS test scores, indicating a lessened membrane function and fertilizing capacity of sperm.

Some phospholipids, such as phosphatidylcholine, could inhibit enterococci β-hemolytic activity by competitive binding of the hemolysin [13]. Because we observed that enterococci β-hemolytic activity was associated with sperm membrane damage, we hypothesized that phosphatidylcholine might inhibit the process. To gain further insight into the possible role of hemolysin on sperm damage, we examined whether hemolysin-induced sperm damage was blocked by phosphatidylcholine. It was found that phosphatidylcholine inhibited hemolysin-induced membrane impairment in a dose-dependent manner (Table 2). When phosphatidylcholine (200 μg/mL) was used, the membrane injury was completely inhibited. Therefore, hemolysin of enterococci lysed not only erythrocytes but also human sperm membrane.

The membrane of sperm were especially damaged on the head, neck and the middle piece of the tail. The membrane in the principal piece of the tail and the end piece of the tail were less damaged than that of the head and the membrane were comparatively intact by electron microscopy. The enriched acrosomal hydrolytic enzymes in the head region of spermatozoa could account for the more significant damage of this region. As we know, the head region of sperm is the main area for sperm to contact with ovum and, therefore, is closely related with fecundation, thereby enterococci were more likely to be related to infectious infertility.

In the present study, there was no significant difference in swelling rate when sperm suspension was incubated with enterococci for only 1 h, but significant difference was found after 3- or 5-h incubation, which indicates that the role of hemolysin on sperm is relative to the incubation time of bacteria. However, it has to be considered that after 3- or 5-h incubation there were excessively more bacteria than after 1 h. The role of hemolysin on sperm relied on its concentration. The concentration of enterococci we used in the present study, which caused damage to sperm membrane, is rarely found *in vivo*, and the incubation time we used was far less than that at the natural condition. The actual situation of *in vivo* infection could be somewhat different. With prolonged infection, the number of bacteria could expand to a level high enough to destroy sperm.

In conclusion, enterococci influenced sperm membrane integrity *in vitro*, and might be mediated by hemolysin. Enterococcus is an important conditioned pathogen with an increasing clinical infection rate. The possible pathogenicity of this opportunistic bacterium should be paid more attention.

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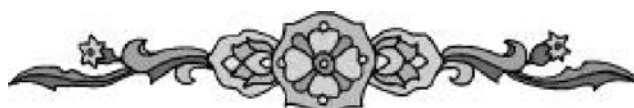

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