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Isolation of Bovine Spermatozoal Components by Physical or Chemical Treatments

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물리 · 화학적 처리에 의한 소 정자세포구성분의 분리

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적 요

정자의 구조와 기능을 이해하기 위해서는 정자세포구성분의 분리가 필요하다. 본 실험은 동결융해된 한우정액에 다양한 물리 · 화학적 처리를 하여 효과적인 정자세포구성분의 분리방법을 확립하고자 실시하였다. 물리적 분리방법으로는 vortexing 처리, 26 gauge needle 또는 strained 26 gauge needle을 1ml 주사기에 부착시킨 후 반복된 pumping, 동결보존액없이 반복된 동결융해처리등을 시행하였다. 또한, 화학적인 분리를 위해 trypsin, dithiothreitol, sodium dodecylsulfate, mercaptoethanol 등을 사용하였다. 모든 처리구중에서 가장 효과적인 정자두부와 미부의 절단은 strained 26 gauge needle이 부착된 주사기를 사용한 반복된 pumping에 의해 얻어졌다. 이러한 처리에 의해 95~100%의 높은 정자구성분의 분리가 이루어졌다. 분리된 정자구성분의 두부표면의 보존여부를 알아보기 위해 250g/ml FITC-UEA 1 염색을 실시하였지만 특별한 두부표면변화는 관찰되지 않았다. 다른 물리적 처리방법들도 높은 정자구성분의 분리결과를 보여주었지만, strained 26 gauge needle를 사용한 방법에 비해서는 분리효율, 시간등 여러면에서 비효율적이었다. 화학적 처리에 의한 정자구성분의 분리결과는 물리적 처리에 비해 효과적이지 못했다. 분리된 정자두부와 미부를 각각 회수하기 위해 sucrose 용액을 2M, 1M, 0.5M, 0.25M 순으로 시험관에 넣은 후 1,000rpm에서 15분간 원심분리한 결과, 1M과 2M의 경계부분에 형성된 정자두부층을 얻을 수 있었다. 정자구성분의 효과적이고 간편한 분리방법이 본 실험에 의해 확립되어졌으며, 위의 방법에 의해 분리, 회수된 정자구성분은 생화학적 연구, 난자활성화기작의 이해등 다양한 응용연구의 기초자료로서 이용될 수 있을 것이다.

(Key words : physical dissection, chemical dissection, bovine spermatozoa)

I. INTRODUCTION

Spermatozoa are highly differentiated haploid cells separating from the body to carry the genetic material to the oocyte during fertilization. The studies on the structure and function of spermatozoal components have helped greatly to understand mammalian fertilization at cellular and molecular terms(Millete *et al.*, 1973; Dale *et al.*, 1985). In particular, oocyte activation could be microdissected by analysing early events occurring in the oocytes treated with various spermatozoal components (Stice and Robl, 1990; Swann, 1990).

The purposes of this study are to establish an effective dissection method for cellular components of spermatozoa and to find an appropri-

ate method for the isolation of spermatozoa components.

Ⅱ. MATERIALS AND METHODS

1. Sperm preparation for dissection of sperm components

Frozen bovine spermatozoa were thawed at 36~37℃ for 20 sec prior to washing in phosphate buffered saline containing 4mg BSA/ml (PBS+BSA). Final sperm pellet was resuspended in 2ml PBA+BSA.

2. Physical dissection of spermatozoa

Two hundred μl of sperm suspension was further diluted by adding 100 μl of PBS+BSA into an Eppendorf tube. The sperm suspensions were vortexed (KMC Vision Co, Korea) for 5 or 10 min. The sperm suspensions were repeatedly pumped with a 26 gauge needle or a 26 gauge needle strained by pliers connected to a 1ml syringe. Also the sperm suspensions were treated by vortexing or pumping with a strained 26 gauge needle connected to a syringe after repeated freezing-thawing without cryoprotectant in −196℃ LN_2.

3. Chemical dissection of spermatozoa

Two hundred μl of sperm suspension was centrifuged at 5,000rpm for 10min. The sperm pellet was treated similarly as reported (Millete *et al.*, 1973) by mixing 400 μl of 0.1 mg/ml trypsin (Fluka, Switzerland), 0.1M dithiothreitol (DTT, BIO-RAD, CA, U.S.A.), 0.01% sodium dodecylsulfate (SDS, BIO-RAD) or 0.1M β-mercaptoethanol (BME, BIO-RAD) solution at 39℃ for 30 min. Four hundred μl of 0.1 mg/ml trypsin was added to the sperm pellet after 0.1M DTT or 0.01% SDS treatment to increase dissection efficiency. The mixture was washed in PBS+BSA by centrifugation before analysis.

4. Isolation of dissected sperm heads and tails

Sucrose (Fluka) solutions of 0.25, 0.5, 1 and 2M were carefully layered 2 ml volume per concentration from top to bottom of a 14 ml cornical tube. And then, the tube was centrifuged at 1,000 rpm for 20 min.

5. Analysis of treatment effectiveness

After dissection of spermatozoal components, the suspensions were analysed to examine the effectiveness of the dissecting methods. Over 200 sperm components were counted under a × 200 Nikon inverted microscope.

6. Sperm surface labelling

Frozen bovine spermatozoa are thawed at 37℃ for 20 sec prior to washing in Ca^{2+} free m-TALP(Yanagimachi, 1982). Washed spermatozoa was swup up in Ca^{2+} free m-TALP for 1h. After swim-up procedure, recovered spermatozoa were washed in PBS+BSA by centrifugation at 5,000 rpm for 10 min. To demonstrate the sperm surface components of the head, either spermatozoa or sperm heads were incubated in 250 μg/ml of fluorescein isothiocyanate conjugated to *Ulex europaeus* I(FITC-UEA 1, Sigma MN, U.S.A) for 1h at room temperature(Lee and Ahuja, 1987). Afterwards unbound FITC-lectins and free FITC molecules were removed by repeated centrifugation in PBS+BSA for over 3 times at 5,000 rpm for 10 min. Spermatozoa were resuspended in a small volume of PBS+BSA, mounted on a glass slide and observed using epifluorescence on a Olympus PM-10 ADS microscope with an appropriate filter for FITC fluorescence. Photographs were taken with Konica GS negative film(ASA 400).

Ⅲ. RESULTS AND DISCUSSION

Various physical methods were used to find a simple and rapid dissection of bovine spermatozoa(Table 1). In each physical treatment groups(Fig. 1) about two hundreds of spermatozoa were counted to evaluate the effectiveness of treatments. The rate of dissected sperm heads were 11.2% in control group which was involved thawing procedure of frozen semen at 37℃ prior to washing in PBS+BSA.

The rates of dissected sperm heads by vortexing treatments were 39.0 and 51.9% for 5 and 10 min, respectively. Vortexing treatments showed little effectiveness on the dissection of sperm heads and tails.

The spermatozoa by repeated pumping with a 26 gauge needle connected to a 1 ml syringe gave 64.4, 95.1 and 100% in 20, 50 and 100 times, respectively. Treatments with needle 26 gauge strained by pliers showed complete dissection when spermatozoa were pumped 50 times. The use of a strained 26 gauge needle was the most simple and effective on the dissection of sperm heads and tails among all treatments including chemical treatments (Tables 1 and 2).

The dissection rate of frozen-thawed spermatozoa three times without cryoprotectants was 23.0%, which was a little higher than that of control group. Also, spermatozoa were vortexed or pumped with a strained 26 gauge needle after freezing-thawing to increase dissection efficiency. However, vortexing treatment for 10 min after freezing-thawing gave lower the dissection rates of 32.2% lower than that (51.9%) of vortexing treatment alone for 10 min. Strained 26 gauge needle treatment after freezing-thawing gave high dissection rates of 95.2 and 100% in 20 and 50 times, respectively. However, many damages were found in sperm heads by freezing-thawing process although strained 26 gauge needle treatment after freezing-th-

Table 1. Effects of physical treatments on the isolation of bovine sperm head and tail

Treatments	Conditions	Total No. of spermatozoa counted	No. of isolated sperm head (%)
Control	–	215	24(11.2)
Vortexing	5 min	205	80(39.0)
	10 min	208	108(51.9)
26 gauge	20 min	202	130(64.4)
needle	50 min	203	193(95.1)
	100 min	232	232(100)
Strained	20 min	200	176(88.0)
needle	50 min	200	200(100)
Freezing & thawing	–	213	49(23.0)
Freezing-thawing & wortex	10 min	202	65(32.2)
Freezing-thawing	20 min	208	198(95.2)
& strained needle	50 min	203	203(100)

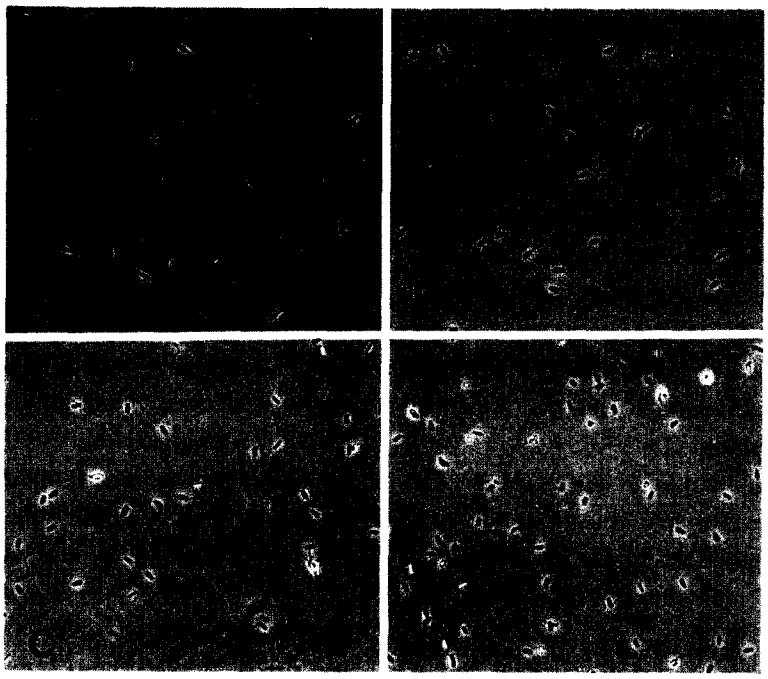

Fig. 1. Bovine spermatozoa after physical treatments. Spermatozoa were processed by a strained 26 gauge needle and sucrose gradient centrifugation.
Intact spermatozoa(control, a) were fragmented by pumping with a strained 26 gauge needle for 20 (b) and 50 times (c), respectively. Isolated sperm heads after sucrose gradient centrifugation were shown in (d). Magnifications are ×200.

awing gave a little higher dissection rates than those of strained 26 gauge needle alone.

Table 2 shows effects of chemical treatments on the isolation of bovine sperm heads and tails (Fig. 2). The dissection rate of trypsin treatment was 25.3% when spermatozoa were exposed by 0.1 mg trypsin for 30 min at 37℃. This result was similar to other reports, bull spermatozoa were resistant to cleavage by trypsin and were cleaved only by treating with a reducing agent(Millette *et al.*, 1973; Pihlaja *et al.*, 1973).

Spermatozoa were incubated with 0.1 or 0.2M DTT in PBS+BSA for 30 min before washing in fresh PBS+BSA and exposure to trypsin. Under these conditions, the dissection rates of spermatozoa were 77.2 and 67.6% in 0.1 and 0.2M DTT, respectively. It indicated no differences between two concentrations of DTT whereas the dissection rates of DTT+trypsin treatment greatly were increased than that of trypsin alone.

Spermatozoa were also incubated with 0.1 or 0.2M BME in PBS+BSA for 30 min before washing in fresh PBS+BSA and exposure to trypsin. The dissection rates of spermatozoa were 78.8 and 75.2% in 0.1 and 0.2M BME, respectively. These results were similar to DTT treatment showing no differences between two concentrations of BME whereas the dissection rates of BME+trypsin treatment were greatly increased than that of trypsin alone. Millette *et*

Table 2. Effects of chemical treatments on the isolation of bovine sperm head and tail[1]

Treatments	Total No. of Spermatozoa counted	No. of isolated sperm head (%)
Control	215	24(11.2)
0.1 mg trypsin	190	48(25.3)
0.1 M DTT & 0.1 mg trypsin	213	164(77.0)
0.2 M DTT & 0.1 mg trypsin	204	138(67.7)
0.01% SDS	209	77(36.8)
0.01% SDS & 0.1 mg trypsin	213	109(51.2)
0.1 M BME & 0.1 mg trypsin	203	160(78.8)
0.2 M BME & 0.1 mg trypsin	218	164(75.2)

1. Cells were treated for 30 min at 39℃ for each chemicals.
2. Abbreviations are DTT, dithiothreitol; SDS, sodium dodecylsulfate; BME, β-mercaptoethanol, respectively.

al. (1973) reported that mouse and rat spermatozoa were cleaved by brief treatment with trypsin to yield free heads and tails while human, guinea pig, and rabbit spermatozoa were cleaved by trypsin only after incubation with BME or DTT. The results obtained in bovine spermatozoa might indicate similar to those of species such as human, guinea pig and rabbit spermatozoa although these species gave higher dissection rates of 90~95%. Also, this might indicate the presence of linkage specificity between head and tail in species.

The dissection rates of SDS alone and SDS+trypsin treatment were 36.8 and 51.2%, respectively. SDS treatments gave low dissection rates in contrast to other detergents treatments. In conclusion, sperm dissection by chemical treatments was less effective than those of physical treatments.

Bull spermatozoa have been cleaved by pronase with extensive fragmentation of spermatozoan tails(Pihlaja *et al.*, 1973). Also, cleavage of sermatozoan heads from tails has been achieved by ultrasonification(Iverson, 1965). However, these techniques did not yield the homogeneous population of cell fragments necessary for precise biochemical analysis.

Dissected bovine spermatozoa were fraction-

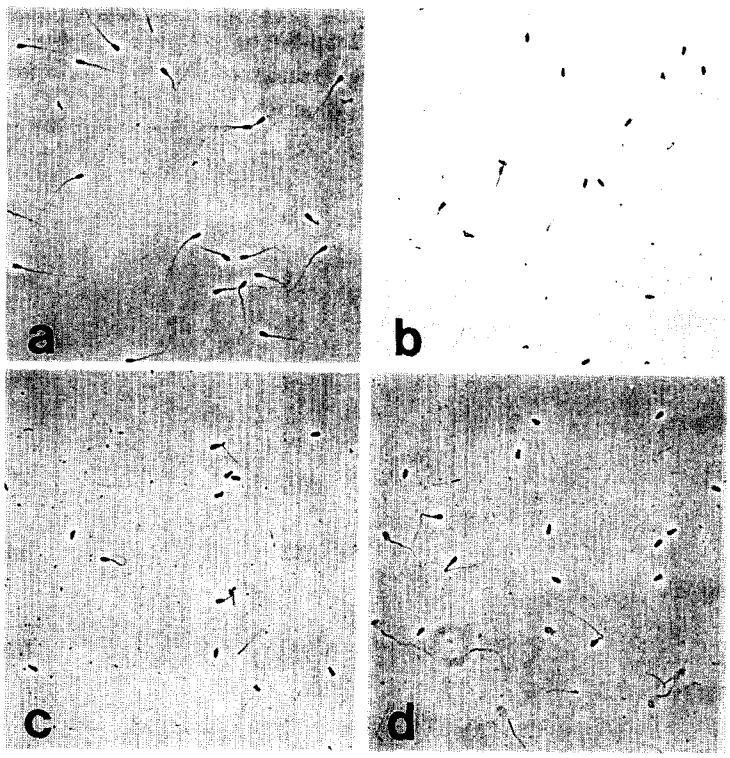

Fig. 2. Bovine spermatozoa after chemical treatments. Intact spermatozoa (control, a) were partially fragmented with 0.01% SDS followed by 0.1 mg/ml trypsin (b), 0.2 M DTT followed by 0.1 mg/ml trypsin(c) and 0.1M BME followed by 0. 1 mg/ml trypsin in PBS for 30 min at 39℃ (d), respectively. Magnifications are ×200.

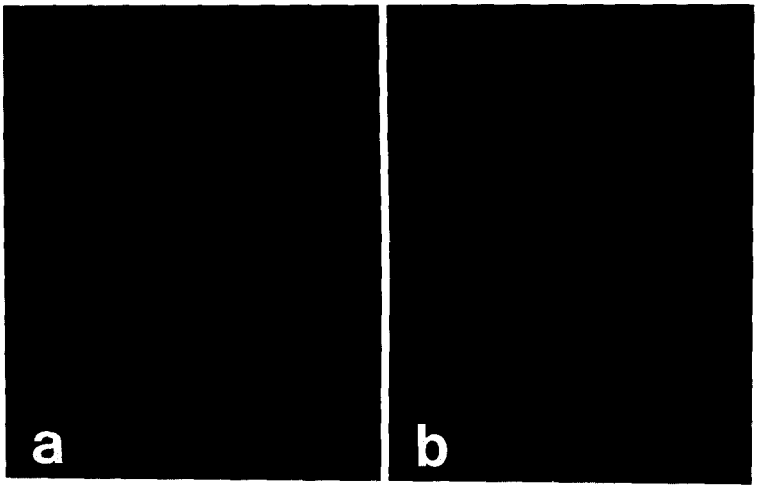

Fig. 3. Lectin binding sites on the surface of intact spermatozoa (a) and isolated sperm head (b) were visualized by using FITC-UEA1. Magnifications are ×400.

ated by sucrose density gradient centrifugation in order to obtain homogenous populations of subcellular components such as sperm heads and tails(Fig. 1). Most of sperm head components could be collected at the upper region of 2M solution although isolated head components were contaminated with a few tails. This result were similar to use of a three step sucrose gradient(2.2, 2.05 and 1.8M) to separate mouse nuclei from tails(Naish *et al.*, 1987). However, the recovery of sperm tail was difficult because of spreading on the layers of 0.25, 0.5 and 1M sucrose solutions. After strained 26 gauge needle treatment, either spermatozoa or sperm heads were incubated in 250 μg/ml of FITC-UEA 1 for 1 h at room temperature to detect the modification of sperm surface components(Lee and Ahuja, 1987). However, Sperm head surface did not modify during such a treatment(Fig. 3).

The most simple and effective dissection method of bovine spermatozoa was established, which used a strained needle connected to a syringe without use of expensive equipment such as ultrasonicator. Also, the recovery of sperm heads by simple sucrose gradient centrifugation could be possible in bovine spermatozoa. Sperm components obtained by these methods could be used in the analysis of DNA-combined protein, the study of sperm decondensation *in vitro*, the microinjection of sperm head into oocyte cytoplasm, the study of acrosomal contents and the analysis of sperm constitution molecules to induce oocyte activation. Also, the improvement of recovery method on sperm tail could be used as basic information for physiological study of flagellar movement *in vitro*.

Ⅳ. SUMMARY

An understanding of the structure and function of mammalian spermatozoa requires the isolation of these components. In this study, frozen-thawed bovine spermatozoa were treated by physical treatments(vortexing, 26 gauge needle, strained 26 gauge needles and freezing-thawing) or chemical treatments(trypsin, dithiothreitol, sodium dodecylsulfate and β-mercaptoethanol) to yield free heads and tails. The most effective treatment was repeated pumping of sperm suspension through a strained 26 gauge needle conneted to a syringe. Spermatozoa by this treatment were mainly broken at the junction of the head and the tail, resulting in 90~100% yields. Also, sperm head surface did not modify during strained 26 gauge needle treatment when either spermatozoa or sperm heads were incubated in 250 μg/ml of FITC-UEA 1 for 1 h at room temperature to detect the modification of sperm surface components. Other physical treatments were less efficient for the breakdown of spermatozoa. The effects of chemical treatments on bovine spermatozoa are not noticeable. Dissected sperm heads and tails should be fractional leading to nearly pure components by sucrose gradient centrifugation at 1,000 rpm for 15 min. The result suggest that the established method may be useful for the biochemical study of spermatozoal components, and the understanding of oocyte activation mechanism either by spermatozoal components during fertilization or microinjection of isolated components.

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