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Determination of indications of intracytoplasmic sperm injection using Film *in situ* Zymography

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Abstract

We tried to evaluate the proteolytic activity of spermatozoa using film *in situ* zymography (FIZ) and decide on the indication of intracytoplasmic sperm injection (ICSI) before *in vitro* fertilization (IVF).

Spermatozoa were obtained from 86 patients including 22 in whom IVF was done in our fertility clinic after fully obtaining informed consent. We classified them into two groups, high rate of fertilization group (A : more than 70%), and low rate of fertilization group (B : under 15%). After all sperm were treated with the percoll gradient method, one drop of sperm (10^5〜10^6 spermatozoa/ml) was placed on the film, and incubated at 37°C for 20 min, then double staining was performed in Ponceau-R and hematoxylin solution. When gelatin was digested enzymatically, the red gelatin stain disappears and becomes transparent.

The activation rate on FIZ was lower in group B than in group A. A distinct correlation was recognized between the activation rate on FIZ and the fertilization rate with conventional IVF. However there was no correlation between the activation rate on FIZ, and concentration, motility or abnormal morphology of sperm. The activation rate on FIZ of the group (more than 15% of sperm penetration assay, SPA) is higher than one of the group (under 15% of SPA).

In this study we demonstrated that the activation of sperm heads by gelatin were low or disappeared in the sperm with low numbers or low motility, and they also showed low fertilization rates in IVF.

These results demonstrated that FIZ is a useful method to determine the need for ICSI in the case of IVF. We conclude that it is also good for a patient psychologically and economically to decide on the necessity of ICSI before IVF.

Introduction

Since Van Steirteghem et al. in 1993 reported an excellent fertilization rate of 64.2% using intracytoplasmic sperm injection (ICSI) in 150 samples in which fertilization by conventional *in-vitro* fertilization (IVF) failed, this method has become widely adopted[1]. However we do not know the long term outcome in infants born after ICSI. There is not conclusive evidence whether this technique is safe in the long term. It is assumed, but not proved that there is no significant difference in incidence of malformation between ICSI and conventional IVF[2-6]. However there is a report that the incidence of malformations rose among infants born after ICSI, for example congenital hypospadias, neural tube defects and obstruction of digestive tract[7]. There is much latent risk in pregnancies with assisted reproductive technology, especially ICSI, because the process chooses a spermatozoon artificially and not naturally. Therefore we must consider indication of ICSI very carefully. However it is a fact that there is no standard examination to choose conventional IVF or

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IVF with ICSI, although there are various reports. For example the sperm penetration assay (SPA) provides a diagnostic tool to assess the biochemical functional events required for capacitation, acrosomal activity, egg penetration and fertilization. The hamster egg-sperm penetration assay is based on the ability of human sperm to fuse with zona pellucida hamster eggs leading to de-condensation of the nucleus. It is also utilized to assess fusiogenic capacity[8)9)]. However this complicated examination uses different species and requires high level of manual skill. The measurement of semen hyaluronidase activity is another way to examine acrosomal enzyme deficiency[10)]. It is very difficult to decide on the indication of ICSI under these examinations. The exact indications of ICSI are unknown, because we lack reliable methods to accurately evaluate sperm fertilization ability. Therefore we tried to develop a good method to examine indication of ICSI. Spermatozoa contain acrosomal enzymes with the proteolytic ability, necessary to pass through the zona pellucida. In this study we tried to evaluate the proteolytic activity of spermatozoa using film *in situ* zymography (FIZ) and determine the suitability of ICSI when we perform IVF[11–16)].

Materials and methods

Spermatozoa were obtained from 86 patients including 22 in whom IVF was performed in our infertility clinic after fully obtaining informed consent (Table 1).

We classified total spermatozoa as either 20,000,000 or less, or more than that, motility rates of either less than 50% or 50% or more and abnormal morphology in less than 30% or 30% or more and evaluated the proteolytic activity of the sperm head using FIZ. Next we classified them into two groups, the high rate of fertilization group (A : more than 70%) and low rate of fertilization group (B : under 15%) and investigated the fertilization rate by IVF.

Sperm penetration assays (SPA) were performed in the above 22 couples after excluding feminine factors. They were selected at random. We classified the results of SPA into two groups, group C (15% or more) and group D (under 15%). We investigated the correlation between the penetration rate of SPA and the activation rate on FIZ.

Film *in situ* zymography (FIZ)

After spermatozoa were treated with the Percoll gradient method, one drop of the sperm solution adjusted to $10^5 \sim 10^6$ spermatozoa/ml was placed on the FIZ film, and drawn across with the edge of a cover slip and incubated at 37°C for 45 minutes, then double staining was performed in Ponceau-R and hematoxylin solution. When gelatin is digested enzymatically, the red gelatin stain disappears and it becomes transparent (Fig. 1)[17)18)]. We observed them with an optical microscope at 400 times magnification, and evaluated the frequency of sperm heads with gelatinolysis (Fig. 2A-D). We counted 2 views for every sample and used the mean value for statistical analysis. The same experimenter observed and evaluated them with a microscope in order to obtain

Table 1 Sperm characteristics

Characteristics	Number of Samples	Mean value
Concentration ($\times 10^6$/ml)		
<20	15	6.7
$\geqq 20$	71	217.1
Motility (%)		
<50	42	22.0
$\geqq 50$	44	70.3
Abnormal morphology (%)		
$\geqq 30$	15	47.3
<30	71	10.2

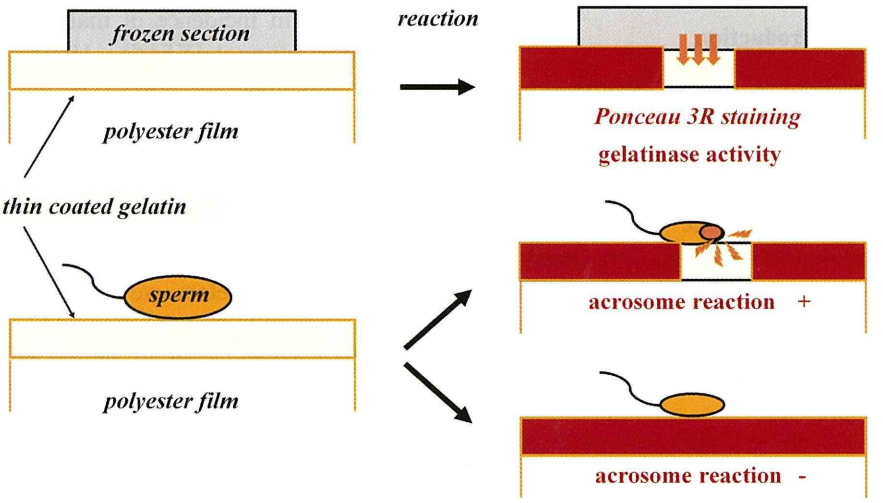

Fig. 1 Principle of FIZ

uniform results.

Sperm penetration assay (SPA)

Fresh liquefied semen (0-2 hours) after ejaculation was warmed for one minute at 37°C, and cooled to room temperature, and buffer was added to double volume. The sample was refrigerated overnight or kept on ice packs. Liquefied semen samples were collected from masturbation and allowed to capacitate overnight in the presence of buffer at 4°C, warmed to 37°C, washed and adjusted to 5 million/ml. Zona pellucida free hamster's ova were prepared by enzymatic digestion, and washed, and then allowed to incubate with sperm solution for 3 hours at 37°C under 5% CO_2[9)19)]. After washing, the ova were examined at 400 times magnifications to determine the penetration of intact spermatozoa.

Statistical Analysis

Data were analyzed by Student's *t*-test for a comparison of means. A p-value of less than 0.05 was considered to indicate a statistically significant difference.

Results

The mean activation rate on FIZ in group B was is 23% and that in group A was 63%. The activation rate of the high fertilization rate group was higher than that of the low fertilization rate group. A distinct correlation ($P<0.001$, $r=0.845$) was recognized between activation rates on FIZ and fertilization rates in conventional IVF (Fig. 3). However there was no correlation between the activation rates on FIZ and concentration, motility, or abnormal morphology of spermatozoa (Fig. 4A-C).

There were 9 couples in whom the activation rate on FIZ was more than 20% and the result of SPA was more than 15%. There were 4 couples in whom the activation rate on FIZ was under 20% and the result of SPA was more than 15%. There were 4 couples in whom the activation rate on FIZ was more than 20% and the result of SPA was under 15%. There were 5 couples in whom the activation rate on FIZ was under 20% and the result

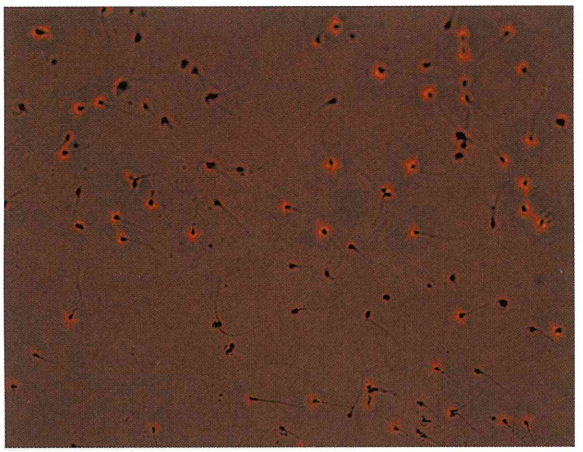

Fig. 2-a

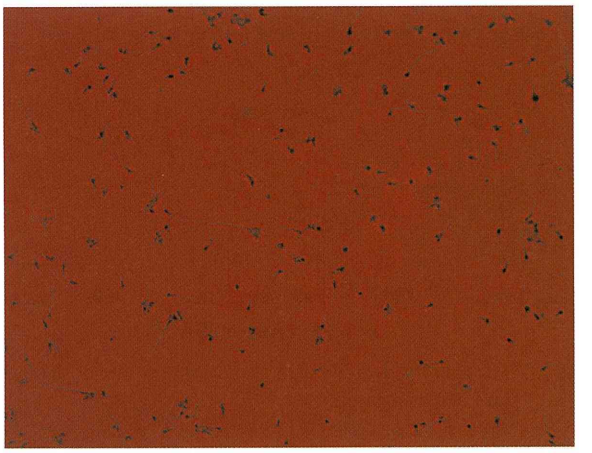

Fig. 2-c

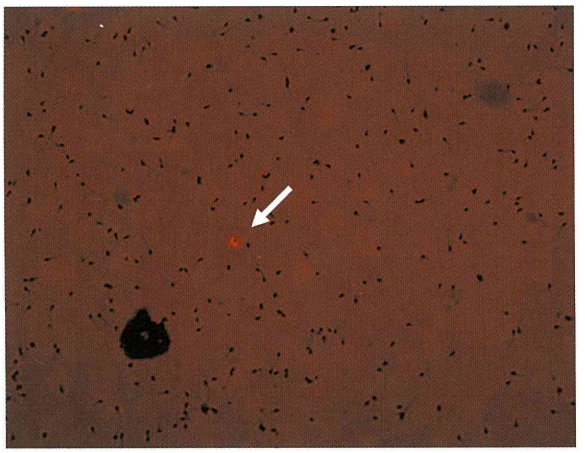

Fig. 2-b

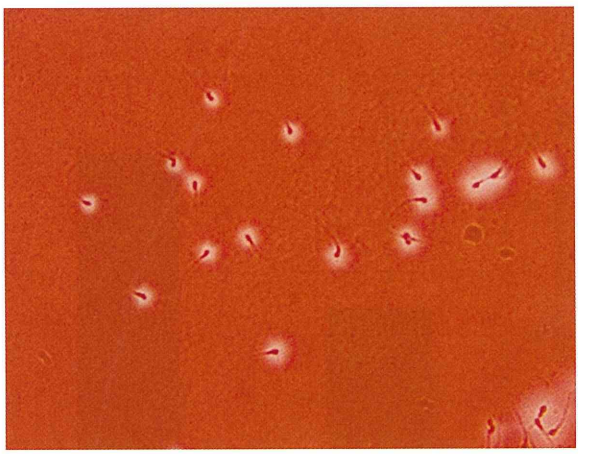

Fig. 2-d

Fig. 2 Typical proteolytic activity of semen by FIZ. Samples were placed on a film coated with gelatin and in situ gelatinolysis was visualized by staining the film with Ponceau-R and Hematoxylin solution. (A) good proteolytic activity, (B) poor proteolytic activity : → indicates gelatinolysis of the head of one spermatozoon, (C) no proteolytic activity, (D) high magnification of good proteolytic activity of sperm.

of SPA was under 15% (Table 2). The mean activation rate on FIZ of group C (more than 15% of SPA) was 69%, and that of group D (under 15% of SPA) was 44%. The activation rate of the group whose SPA was high was higher than that of the low fertilization rate group. However there was no correlation between the activation rate on FIZ and the penetration rate of SPA (Fig. 5).

Discussion

Spermatozoa pass through follicular cells surrounding the oocyte and reach the zona pellucida. They can pass through the zona pellucida after capacitation and the acrosome reaction have occurred. Many types of enzymes, including serine proteases such as acrosin in the acrosome substances, are needed to penetrate the corona radiata barrier and help the spermatozoon across the zona pellucida. However the detailed mechanism about these molecules is not yet elucidated. Therefore we used FIZ which detects protease activity of enzymes such as matrix metalloproteinases (MMPs), and trypsin, and to examine the fertilization ability of spermatozoa.

We clarified that the fertilization rate in conventional IVF was low when FIZ results showed a decline of the gelatinolytic activation of the heads of spermatozoa.

It was suggested that there is a connection between the activation rate on FIZ and fertilization rate in comparison with SPA. When high activation rate on FIZ was recognized, SPA was also thought to be high. These results demonstrated that FIZ is useful in the selection of ICSI in the cases of IVF. We think that it is also good

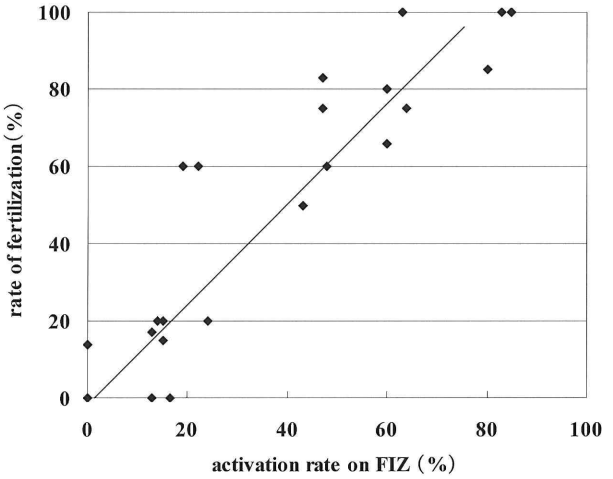

Fig. 3 The relationship between the activation rate on FIZ and rate of fertilization

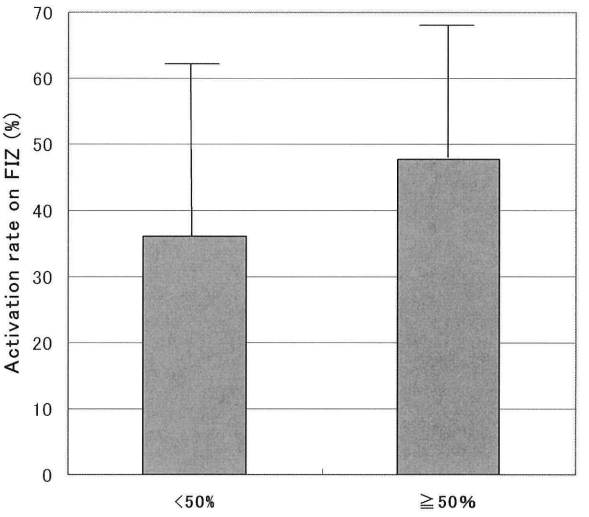

Fig. 4-b

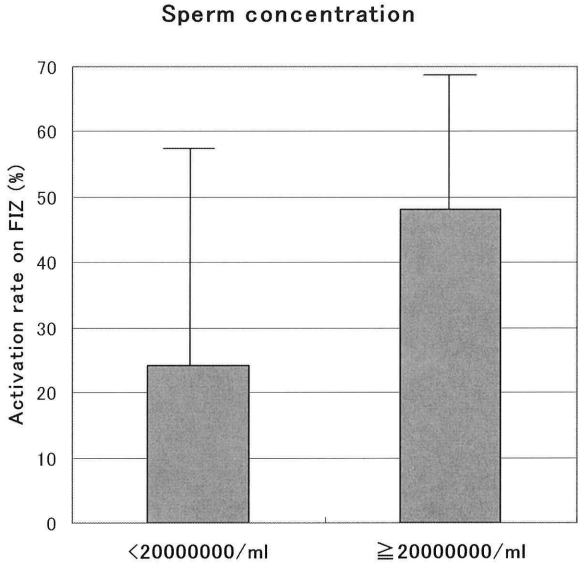

Fig. 4-a

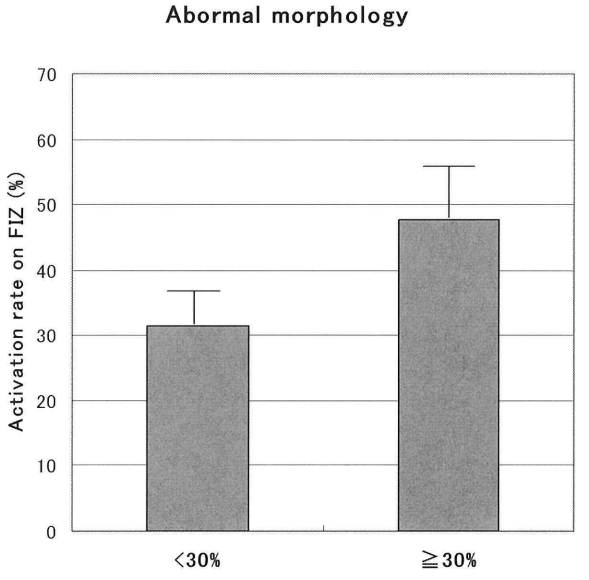

Fig. 4-c

Fig. 4 Comparison of the activation rate on FIZ in classified categories of sperm concentration (A), motility (B) and abnormal morphology (C)

Table 2 Results of the penetration rate of SPA and the activation rate on FIZ on the same samples

	FIZ (20%<)	FIZ (20%>)
SPA (15%<)	9/13	4/13
SPA (15%>)	4/9	5/9

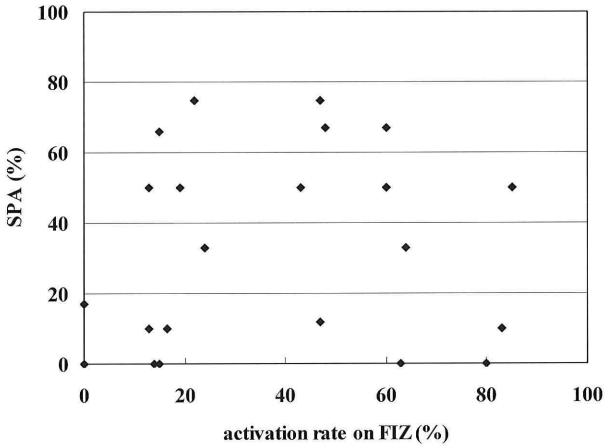

Fig. 5 The relationship between the activation rate on FIZ and SPA

for a patient psychologically and economically to decide on the necessity of ICSI before IVF. FIZ is a useful method of judging the necessity of ICSI before IVF, bur further improvement of this method, particularly the incubation time and the number of spermatozoa and volume of sperm solution applied on the film is required, although this procedure is simple, easy and economic. We will perform ICSI, when the activation rate on FIZ is low, especially under 20%. In this study we examined only 22 couples in whom IVF was done in our infertility clinic after fully obtaining informed consent. Therefore we will continue to accumulate cases in the future.

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Film *in situ* Zymography を用いた intracytoplasmic sperm injection の適応に関する検討

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【要旨】 体外受精を施行する際の intracytoplasmic sperm injection (ICSI) の選択を考える上で、受精能の検討は非常に重要である。しかし精子受精能の評価に関しては現在のところ一定の見解はなく、信頼に足る評価方法もない。そこで我々は FIZ (Film *in situ* Zymography) を用いて精子頭部のゼラチナーゼ活性を検出し、これが精子の受精能の判定に有用か否か検討した。承諾を得られた *In vitro* fertilization (IVF) 周期の症例 22 例を含む 86 例に対し、精子数、運動率、奇形率を求めた。次に FIZ を用いて精子のゼラチナーゼ活性率を算出した。10^5〜10^6 sperm/ml の精子 0.1 ml をフィルム上に滴下後、37°C で 45 分間インキュベートし、ポンソー3R およびヘマトキシレンにて染色した。また IVF 周期の受精率と FIZ の活性率を比較検討した。また、IVF 周期の 22 症例では、ハムスターテストを施行、A 群 (15% 以上)、B 群 (15% 未満) にクラス化し、FIZ の活性率を検討した。統計学的な処理にて精子数、運動率、奇形率と FIZ の活性率に相関は認められなかったが、IVF 時の受精率と FIZ の活性率には相関が認められた。受精率が低い群に比べて、高い群では FIZ の活性率が有意に高かった。また、ハムスターテストと FIZ の活性率に相関関係は認められなかったが、ハムスターテストが 15% 以上の群では、FIZ 活性率は平均 69% (9/13 例)、ハムスターテストが 15% 以下の群では、FIZ 活性率は 44% (4/9 例) と、ハムスターテストが高い群で FIZ 活性率は高かった。今回我々は、精子頭部のゼラチナーゼ活性と conventional IVF における受精率の相関を確認した。このことより ICSI の適応の選択に FIZ が有用であると思われた。

〈キーワード〉 film *in situ* zymography (FIZ)、体外受精、顕微授精