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The Effects of Vero Cells Coculturing on the Motility of Human Testicular Spermatozoa in an Intracytoplasmic Sperm Injection Program

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정자직접주입술에 있어 Vero Cells 공배양이 인간 정소정자의 운동성에 미치는 영향에 관한 연구

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= 국문초록 =

폐색성 혹은 비폐색성 무정자증에서 부정소 정자채취법 등이 부적절하다고 여겨질때는 정소 조직을 일부 절제하여 그 조직으로부터 정자를 직접 채취하게 되는데 일반적으로 이렇게 정소로부터 추출한 정소정자는 운동성이 전혀 없거나 매우 약한 운동성을 보이는 경우가 많다. 본 연구의 목적은 이러한 정소정자를 Vero cell과 공배양을 시킴으로써 운동성을 획득시키거나 향상시키고 이를 수정시키는 시기까지 지속시킴으로써 정소정자추출술 (TESE)을 시행하는 환자나 의료진들에게 보다 편안하고 융통성있는 시간대를 부여하고, 아울러 정자직접주입술 (ICSI)을 보다 용이하게 하여 성공적인 수정률과 임신율을 얻음에 있다. 또한 ICSI를 시행한 후, 운동성이 향상된 잉여의 정소정자를 냉동보존함으로써 차후에 TESE을 다시 시행치않고도 시험관 아기 시술을 시도할 수 있는 부가적인 잇점도 있다고 할 수 있다.

대상환자군은 정관폐색증 (n=11) 혹은 비정관폐색증 (n=2)을 보이는 13명의 무정자증의 남성불임 환자였으며 난자회수예정일 3일전에 TESE를 시행하여 정소정자를 얻은 후 이를 정자직접주입술이 시행되는 당일까지 Vero cell과 공배양을 실시하였다. Vero cell과의 공배양에 의하여 운동성이 있는 정소정자의 수는 공배양전과 비교하여 평균 3.3배가 증가하였으며, 특히 공배양전에 운동성이 있는 정소정자의 수가 50,000/ml 이하의 미약한 운동성만을 보였던 경우 (n=5)에는 공배양 후에 운동성이 있는 정소정자 수의 평균증가율이 7.7배였다. 공배양전 정자운동성이 전혀 없었던 2례의 비정관폐색증환자중 3일간의 공배양을 통하여 1례에서 운동성을 획득한 정소정자를 얻을 수 있었으며 (14,300/ml), 정자직접주입술을 통하여 성공적인 수정 및 임신에 도달할 수 있었다. Vero cell과 공배양을 하고 ICSI했던 결과, 평균 수정률은 75.0% 이었으며 임신율은 61.5%였다.

Key Words: 폐색성, 비폐색성무정자증, Vero cell, TESE, ICSI

INTRODUCTION

Since the first report by Schoysman *et al.* (1993) of a successful pregnancy using testicular spermatozoa through testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI), the use of TESE-ICSI or tes-

ticular sperm aspiration (TESA) (Craft *et al.*, 1995)-ICSI has become one of the main treatment options in many IVF centers. However, the motility of spermatozoa retrieved from the testis is usually very low, and in some cases, only non-motile spermatozoa (weakly-shaking spermatozoa) are retrieved (Liu *et al.*, 1996). Because of this poor motility, selection of motile testicular sperm for ICSI can sometimes become difficult and consequently may result in poor fertilization rates (Edirisinghe *et al.*, 1996). For this reason, several researchers have recently tried to enhance the motility of testicular sperm using various culture systems. In 1996, Zhu and coworkers reported that testicular sperm motility was improved after *in vitro* culture using standard IVF culture medium (Medicult a/s, Copenhagen, Denmark). Liu and colleagues (1996) also reported a similar result with B2 medium in the same year, and they suggested that testicular spermatozoa in patients with obstructive azoospermia could be retrieved 3 to 4 days prior to the day of oocyte retrieval. However, it was noted that testicular spermatozoa in patients with non-obstructive azoospermia should be retrieved and injected on the same day of oocyte retrieval, because the results of *in vitro* culture using testicular spermatozoa from non-obstructive patients culturing in B2 medium were unpredictable.

We felt that the motility of testicular spermatozoa could be increased even more if a coculture system was employed instead of a single culture system. In fact, many researchers have reported the positive effects of coculture on the motility of human ejaculated or epididymal spermatozoa using epididymal epithelium cells (Moore *et al.*, 1992; Akhondi *et al.*, 1997), oviductal epithelium cells (Kervancioglu *et al.*, 1994; Morales *et al.*, 1996), uterine epithelium cells (Fusi *et al.*, 1994; Pacey *et al.*, 1995), or Vero cells (Wetzels *et al.*, 1991; Pearlstone *et al.*, 1993; Chen *et al.*, 1994). However, it seems that there has previously been no report concerning the clinical use of testicular spermatozoa after coculture with feeder cells, including Vero cells, in an ICSI program. Therefore, we attempted to coculture spermatozoa with Vero monolayer cells for several days to observe whether or not there was an increase in the motile testicular spermatozoa count (MTSC). After coculturing, ICSI was performed with cultured testicular spermatozoa, and the effects of Vero cell coculture on the rates of fertilization and pregnancy were determined.

MATERIALS and METHODS

1. Patient recruitment

In 13 infertile couples with obstructive or non-obstructive azoospermia, TESE-ICSI was performed between December of 1996 and May of 1997 at the Infertility Medical Center of Cha General Hospital. All male patients were evaluated by genital examination, hormonal assessment, and ultrasound to determine testicular volume. Also, histological examinations of testicular biopsy specimens were performed during the diagnostic work-up. Cases in which spermatozoa were not obtained after TESE were excluded from the study.

2. Vero cells preparation

Vero cells were commercially obtained (RIKEN Cell Bank, Japan) and propagated according to the recommended protocol. For experimental use, cells maintained in culture flasks in TCM 199 medium with 10% FBS were dissociated using a trypsin-ethylenediaminetetraacetic acid solution (Gibco, Co., USA), washed with calcium magnesium ion free phosphate buffered saline, and then seeded into a micro drop (100 μl) at a density of 25,000/ml. The cells were then allowed to grow to confluence, with routine changes of TCM199 medium with 10% FBS every 3 days, which were then used within 2 days.

3. Testicular sperm preparation and coculture with Vero cells

It was determined from previous experiments that 3 days of coculturing testicular spematozoa with Vero cells was the optimal culture duration for improving sperm motility. Therefore, testicular tissue samplings were performed several days prior to the scheduled day of oocyte retrieval. The surgical techniques of TESE have been described previously (Cha *et al.*, 1997). Briefly, the isolated testicular tissue was minced with a surgical blade in a Petri dish (3002, Falcon, USA) containing 1.0 ml of Ham's F-10 medium with 10% FBS to obtain a suspension of spermatozoa. The suspension was then washed two times (300 g, 2 min) with Ham's F-10 medium with 10% FBS. After a second centrifugation, the supernatant was removed and the sperm pellet was mixed briefly using a vortex (3secs). The amount of sperm suspension was adjusted to about 40 to 50 μl, and each 10 μl of sperm suspension was then transferred to a 100 μl Vero cell droplet under sterile paraffin oil (BDH Co, United Kingdom) in a Petri dish (3002, Falcon, USA).

All the specimens were investigated for MTSC on the day of testicular sperm retrieval and on the day of ICSI. Testicular spermatozoa were first incubated 2hrs in a 5% CO_2, 37℃ incubator before examing the initial MTSC. Testicular spermatozoa were then cocultured with Vero cells until the day of oocyte retrieval.

4. Ovarian hyperstimulation

Ovarian stimulation was performed with hMG (Pergonal; Serono, Australia) and FSH (Metrodin; Serono, Australia) in combination with leuprolide acetate (Lucrin; Abbott, Australia) or buserelin acetate (Suprefact; Hoechst, Brussels, Belgium), using a long protocol. Oocytes were collected by transvaginal ultrasound puncture 36 hours after administration of 10,000 IU hCG (Profasi; Serono, Australia).

5. Intracytoplasmic sperm injection procedure

Cumulus and corona cells were removed by hyaluronidase after oocyte retrieval, and only metaphase II oocytes were used for ICSI. Holding and sperm injection pipettes were made from microcapillary pipettes (Drummond Co., USA) in our laboratory using a micro puller (Sutter instrument Co, USA) and a micro forge (Alkatel Co., France). The inner and outer diameter of the injection micro pipette used for ICSI was 5 to 6 μm and 7 to 8 μm, respectively. The testicular sperm suspension was introduced into Earle's Balanced Salt Solution (EBSS, Gibco, USA) with 0.3% (w/v) bovine serum albumin. Motile spermatozoa were segregated from non-motile spermatozoa in the EBSS medium drop and were then moved to a drop of 5% Polyvinyl pyrrolidone (PVP, Sigma, USA). The motile spermatozoon was washed several times in the PVP drop, and then a single spermatozoon was chosen and immobilized. The immobilized spermatozoon was then moved to EBSS medium containing the oocytes, and was injected carefully into the ooplasm.

6. Assessment of fertilization and pregnancy

About 16 to 18 hours after sperm injection, the oocytes were observed under an inverted microscope and fertilization was considered normal when two clearly distinct pronuclei were seen. Embryos were transferred to the fallopian tube (s) and/or uterus depending on the patient's condition. Pregnancy was confirmed by detecting increasing serum β-hCG concentrations for 10 days following embryo transfer.

RESULTS

Overall, 13 cycles of ICSI were attempted using testicular spermatozoa cocultured with Vero cells in vitro for 2 to 4 days. Eleven cycles were performed using sperm from patients with obstructive azoospermia and two cycles were performed using sperm from patients with non-obstructive azoospermia patients. The mean age of the male and female patients was 36.5 years (ranging from 30 to 42) and 32.5 years (ranging from 28 to 38), respectively. The mean$\pm$SD values for serum FSH and testosterone concentrations in the male patients were 11.7$\pm$10.3 IU/l and 5.1$\pm$2.5 ng/ml, respectively.

Testicular spermatozoa from 8 patients were cocultured with Vero cells for 3 days. The mean MTSC after each 2, 3, and 4 days coculture with Vero cells was increased, up to 3.1, 3.3, and 2.9 times the initial MTSC respectively. According to the culture duration, no difference in the improvement of the MTSC between 2,3 or 4 days coculture with Vero cells was found.

On the other hand, the obstructive group with very low MTSC (less than 50,000/ml) showed the best improvement, with a mean final MTSC count 7.7 times that of the initial motile sperm count. Also, in the two patients with non-obstuctive azoospermia (0% initial motility), though one patient had no improvement in his final MTSC, another patient showed several motile testicular spermatozoa (14.3 $\times$ 10^4/ml) after 3 days of coculture with Vero cells (Table 1).

In 13 couples, ICSI was performed using cocultured testicular spermatozoa. A total of 86 metaphase II oocytes were microinjected, of which 84 oocytes survived (97.7%) and 63 oocytes fertilized (75.0%) normally. Embryo transfers were successful in all 13 cycles, and we achieved 8 pregnancies (61.5%). Eight of the nine patients whose female partner showed no severe infertility became pregnant, but all 4 patients whose female partner showed some infertility had failed pregnancies. Table 2 shows the details.

DISCUSSION

Extraction or aspiration of testicular sperm from the testis and its use in ICSI to achieve successful fertilization and pregnancy has become one of the main treatment options for

Table 1. The effects of Vero cells coculture on the motility of testicular spermatozoa

Patient	Culture duration (days)	Motile sperm count ($\times 10^4$/ml)		Initial /coculture	Category
		initial	coculture		
LJK	2	7.1	32.1	4.5	obstructive
YDJ	2	60.7	178.6	2.9	obstructive
SSD	3	0.0	0.0	0.0	non-obstructive
YJH	3	0.0	14.3	14.3	non-obstructive
CDH	3	2.3	28.6	12.4	obstructive
KDS	3	5.0	39.6	7.9	obstructive
OHJ	3	14.2	46.4	3.3	obstructive
OSJ	3	25.0	92.9	3.7	obstructive
KKT	3	50.0	161.2	3.2	obstructive
LGC	3	203.6	535.7	2.6	obstructive
CBT	4	3.2	24.3	7.6	obstructive
LSI	4	3.6	14.3	4.0	obstructive
KJJ	4	29.0	98.5	3.4	obstructive

Table 2. Outcome of intracytoplasmic sperm injection and embryo transfer

Patient	No. of oocytes				Pregnancy	Female factor
	retrieved	injected	survived	fertilized		
LJK	15	12	11	9	Yes	Normal
YDJ	14	6	6	5	Yes	Normal
SSD	11	7	7	7	Yes	Normal
YJH	5	5	5	5	Yes	Normal
CDH	10	8	8	7	Yes	Normal
KDS	12	8	7	6	No	Normal
OHJ	9	6	6	4	Yes	Normal
OSJ	12	9	9	6	Yes	Normal
KKT	21	12	12	9	Yes	Normal
LGC	2	2	2	1	No	Ovaian factor
CBT	1	1	1	1	No	Uterine factor
LSI	8	4	4	2	No	Uterine factor
KJJ	10	6	6	1	No	Pelvic adhesion, Tubal factor
Total	130	86 (66.26%)	84 (97.7%)	63 (75.0%)	61.5%	

male infertility (Schoysman *et al.*, 1994; Silber *et al.*, 1995; Tucker *et al.*, 1995; Craft *et al.*, 1995). However, fertilization rates using testicular spermatozoa are not much higher than with ejaculated spermatozoa because of their poor motility (Nagy *et al.*, 1995).

Since 1996, some researchers have investigated the effects of testicular spermatozoa culture for improving motility. Zhu and coworkers (1996) revealed that the culture of freshly collected testicular spermatozoa in vitro for 3 days improved motility progressively. Ghunaim and his colleagues also reported in 1997 that motility of testicular sperm from obstructive patients was improved after a 24 hour incubation period in culture medium.

Previous experiments performed by other researchers only used simple culture method, thus we felt that the introduction of a coculture system was needed to improve the motility of testicular spermatozoa from not only obstuctive azoospermia patients, but also non-obstructive azoospermia patients as well. Although many reports have revealed the positive effects of coculture on the motility of human ejaculated or epididymal spermatozoa using various epithelium cells, there seems to be no report concerning the coculturing of human testicular spermatozoa with any kind of monolayer cells. We chose Vero cells among the various monolayer cell lines since the cells had several advantages compared to other cell lines. Bongso and coworkers (1991) reported previously that Vero cells produce growth factors (IGF, TGFβ1), glycoproteins (17 kDa, 25 kDa, PP5, PP14) and an antioxidant (taurine), and may remove toxic materials from the culture media. Moreover, Vero cells are much easier to handle compared with other helper cells, and are commercially available.

Coculture duration was considered one of the most important factors in designing our experiments. Zhu *et al.* (1996) reported that they observed the highest motility of sperm extracted or aspirated from testicular tissue after 3 days of in vitro culture in simple medium. In our preliminary experiments, we also obtained the highest number of motile testicular sperm

after 3 days coculture with Vero cells. Therefore, it was determined that the optimal testicular sperm retrieval day should be 3 days before the day of oocyte retrieval, but we also found that culture duration could be shortened or extended, because the sperm motility was elevated and maintained from 2 to 6 days of coculturing.

By coculturing testicular spermatozoa with Vero cells, the final MTSC was increased 3.3 times compared with the initial MTSC. The group with very low initial MTSC (less than 50,000/ml) (n=5) showed the best improvement, with a mean final MTSC 7.7 times that of the initial MTSC. As reported previously by others (Zhu *et al.*, 1996; Liu *et al.*, 1996), we achieved better improvement of motility in patients with obstructive azoospermia. Additionally, in one non-obstructive case, the MTSC increased from 0 to 14.3×10^4/ml after 3 days of Vero cells coculture. Other researchers (Zhu *et al.*, 1996; Liu *et al.*, 1996) reported no or very little effect of culture in vitro on the testicular sperm motility obtained from patients with non-obstructive azoospermia. Although the study numbers for non-obstructive patients were very small in this experiment, making it difficult to compare our results with others, we definitely obtained motile sperm in non-obstructive patients. The viability of sperm from patients with non-obstructive azoospermia may be correlated with the effects of Vero cell coculture (ie., growth factors, components associated with acquiring motility, or other unknown factors). We are now undergoing a study involving the compensations between the physiology of sperm from patients non-obstructive and obstructive azoospermia. We cryopreserved supernumerary sperm for futher use, but further investigation is needed to assess whether Vero cell coculturing can also improve the recovery of the number of motile testicular spermatozoa after cryopreservation.

CONCLUSION

In conclusion, coculturing testicular spermatozoa with Vero cells in vitro can increase the motility of fresh testicular spermatozoa from patients with obstructive azoospermia as well as from patients with non-obstructive azoospermia. Specifically, three days of in vitro coculture with Vero cell monolayers is the optimal incubation time for improving motility (up to 3.3 times (mean) with the initial motility). The improvement in motility after coculturing facilitates the selection of motile spermatozoa for ICSI and also shortens the time required for the ICSI procedure. Normal fertililzation (75.0%) and high pregnancy (61.5%) rates were achieved after coculturing testicular spermatozoa with Vero cells. Since, testicular biopsies were performed 3 days before aspiration, therefore Vero cell coculturing gives time flexibility to the physician and patient for the testis biopsy procedure. The supernumerary motile testicular spermatozoa cocultured with Vero cells can be saved for another ICSI cycle through cryopreservation techniques.

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