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박사학위 청구논문

Characterization of the factors influencing on embryo quality and outcomes in human assisted reproductive technologies

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인준서

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논문개요

난임 치료를 위해 난자와 배아를 다루는 보조생식술(Assisted reproductive technologies, ART)는, 1978년 최초 시험관 아기인 루이스 브라운의 출생 이후 지속적으로 개선되고 발전하고 있다. 기본적으로 체외수정 (In vitro fertilization, IVF), 배아의 배양, 그리고 배아이식(Embryo transfer, ET)을 포함하고 있는 보조생식술은, 과배란유도(Controlled ovarian hyperstimulation, COH), 착상전유전진단(Preimplantation genetic diagnosis, PGD), 그리고 대리모시술(Surrogacy) 등과 같이 폭넓은 분야를 모두 포함하고 있다. 본 연구는 인간 보조생식술에서 배아의 질과 임신결과에 미치는 요인들의 특성을 규명하기 위하여 수행되었다.

제 1장에서는 ART 중에서 수정 현상, 배양 조건과 이식 요인을 중심으로 세포질내정자수입술(intracytoplasmic sperm injection; ICSI), 고환정자채취술(Testicular sperm extraction, TESE), 포배기배아 배양 및 이식, 배양환경 및 배양기 등에 대한 기반적 접근과 본 연구의 개요를 설명하였다. 제 2장에서는 대표적인 수정 방법인 일반 수정(conventional in vitro fertilization)과 세포질내정자주입술의 효용성을 비교하였다. 초기 세포질내정자주입술은 남성난임 요인을 치료하기 위해 개발되었다. 그 후 세포질내정자주입술의 시행 범위가 확대되어 남성난임 요인이 아닌 경우에도 적용되었다. 그러나 비남성난임 요인을 가진 경우, 세포질내정자주입술의 효용성에 대한 충분한 근거는 아직 규명되지 않았다. 이에 2장에서는 비남성난임 요인에 대한 세포질내정자주입술의 효용성을 확인하기 위하여, 분할수정(split insemination) 시술에서 일반수정과 세포질내정자주입술의 결과를 비교

하였다. 수정률과 배아의 질은 일반수정과 세포질내정자주입술에서 차이가 없었다. 따라서, 비남성난임요인의 경우 세포질내정자주입술의 시행이 수정상의 문제가 없는 경우 꼭 필요하지 않을 것으로 사료된다.

제 3장에서는 획기적인 보조생식술 기법 중 하나인 고환조직정자채취술의 효용성을 분석하였다. 고환조직정자채취술과 세포질내정자주입술을 함께 시행함으로써, 무정자증 환자에서도 성공적인 임신결과를 얻을 수 있다고 보고되고 있다. 많은 연구들에서 포배기배아 배양 및 이식을 통한 임신율의 향상이 보고되고 있다. 따라서 3장에서는 고환조직정자채취술을 통하여 회수된 고환정자를 사용하여 발달된 배아와 사정된 정자를 사용하여 발달된 배아의 포배기배아 형성과 포배기배아의 질을 비교하여 그 효용성을 확인하였다. 포배기배아 형성률과 질은 두 군간에 유의한 차이 없었다. 이는 정자의 수정 이후 배발생에 있어서의 발생능력은 세정관 내 정자도 사정된 정자와 동일함을 의미한다. 종합하여 보면 적절한 수의 난자를 얻을 수 있고, 고환조직정자채취술을 통하여 정자를 얻을 수 있다면, 무정자증 환자에서도 포배기배아 배양 및 이식주기를 고려해 볼 수 있을 것이라고 사료된다.

제 4장에서는 배아발달을 최적화하고, 이식할 수 있는 우수한 배아를 얻기 위한 배양조건에 관한 연구를 시행하였다. 배양 환경에서 가장 중요한 것은 배양기 내 산소농도, pH, 및 온도의 안정성 등 이다. 배양기의 산소농도, pH, 온도 등의 조건을 조절함으로써 배아배양 환경을 최적화 시킬 수 있다. 4장에서는 배양조건 중 산소농도가 배아의 질과 임신률에 미치는 영향을 알아보았다. 산소농도

는 생리학적 농도 (5 % O_2, 5 % CO_2, 90 % N_2)와 대기 농도(공기중의 20% O_2와 균형을 이루는 5% CO_2)로 나누어 각각 배아를 배양하였다. 연구결과, 두 군간 수정 및 배아의 질에서 유의한 차이는 없었다. 그러나, 생리학적 산소농도(5 % O_2)에서 배양한 배아의 착상률은 대기 산소농도에서 배양한 배아의 착상률에 비하여 통계적으로 유의하게 높게 나타났다. 본 연구결과 산소농도에 따른 수정과 배아의 질에 차이가 없었으나 생리학적 산소농도(5 % O_2)의 배양 조건이 체외수정의 성공률을 높일 수 있는 것으로 사료된다.

제 5장에서는 배양액으로의 온도 전달과 배양접시와의 밀착 정도가 서로 다른 배양기에 따라 배아의 질과 임상결과에 미치는 영향에 관하여 연구를 진행하였다. 전통적으로 사용되어온 표준형 박스 형태의 배양기(Standard Box-Type incubators)와 건조형 벤치탑 배양기(Benchtop incubators)의 두 가지 유형의 배양기에서 배아를 배양한 후 이식한 결과를 비교하였다. 연구결과 배아의 질을 제외한 임상결과는 두 군간 차이가 없었다. 배아의 질은 건조형 벤치탑 배양기에서 배양했을 통계적으로 유의하게 더 우수한 것으로 나타났다. 이것은 건조형 벤치탑 배양기가 추구하는 빠른 가스농도와 온도의 회복 때문인 것으로 추정된다. 한편 질적으로 동일하다고 판단된 배아를 이식한 후 착상율에서 유의한 차이는 없었다. 따라서 배양기의 환경 배양 환경 회복 정도를 분석하여 어떤 유형의 배양기를 사용하는 것이 좋은 가를 판단하여 사용할 필요가 있다.

이와 같이, 수많은 보조생식술이 체외수정 시술의 결과에 직접 또는 간접적으로 영향을 미친다. 따라서 성공적인 결과를 얻기 위해서는 철저한 조사와 신중

한 판단을 통한 보조생식술의 결정과 지속적인 배양환경의 관리 및 시술이 필요할 것으로 사료된다.

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Chapter 1

The Field of Assisted Reproductive Technologies

Assisted reproductive technologies (ARTs) have been continuously improved since the birth of Louise Brown in 1978. The field of ARTs, basically involving in vitro fertilization, embryo culture, and embryo transfer, has developed and expanded. Intracytoplasmic sperm injection (ICSI) was initially developed to treat male factor infertility and has significantly improved fertilization rates (Palermo et al., 1992). Palermo et al. (1992) reported the first series of live births following ICSI for couples with severely impaired sperm characteristics, in whom IVF and subzonal insemination had failed previously (Huang and Rosenwarks, 2014). ICSI has the potential to overcome possible fertilization problems when semen characteristics are poor. Recently ICSI is usually performed when retrieval of small number of oocytes, poor quality oocytes, previous total fertilization failure, poor fertilization, advanced maternal age, in vitro maturation (IVM), preimplantation genetic diagnosis (PGD) and fertilization of frozen-thawed oocytes (Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2012). Fertilization failure is very stressful situation undergoing IVF-ET cycles, so ICSI usually has been performed not only male factor cases but also mild or non-male factors. When total fertilization failure occurs, ET will be cancelled consequentially and there is no chance of pregnancy. For the prevention of fertilization failure, ICSI is usually applied also in cases without male factors, although effectiveness of ICSI is not proved perfectly in non-male factor cases.

ICSI can overcome the severe male factors even though azoospermia. ICSI using spermatozoa from directly obtained testicular tissue has been well established so far, and studies have been reported with acceptable fertilization and pregnancy outcomes (Devroey et al., 1994; Schoysman et al., 1993; Silber et al., 1995; 1996)). ICSI combined with testicular sperm extraction (TESE) has been evaluated to be an effective treatment method for both obstructive and non-obstructive azoospermia and TESE-ICSI has become a common procedure in ARTs (Schill et al., 2003). The spermatozoa recovery rate in TESE has been reported from 40%-70%. Especially, the recovery rates are lower in non-obstructive azoospermia (NOA) than obstructive azoospermia (OA) because of defective spermatogenesis in NOA cases (Mansour et al., 1996; Mercan et al., 2000; Friedler et al., 1997, Rosenlund et al., 1998). A number of studies have reported that clinical outcomes of IVF-ET depended on quality of retrieved sperm. They showed that blastocyst formation was low when sperm quality was poor (Janny and Menezo, 1994, Shoukir et al., 1998; Palermo et al., 1999). Some studies have compared clinical outcomes between ejaculated sperm and testicular sperm and, reported that fertilization rate and blastocyst formation rate using testicular sperm were higher than those using ejaculated sperm (Balaban et al., 2001; Rossi-Ferragut et al., 2003). Recently other studies was reported that the ability of testicular sperm to support embryonic development was comparable to that of ejaculated or epididymal sperm (Nilsson et al., 2007; Naru et al., 2008; Braga et al., 2013, Xie et al., 2014). However, it is unclear that the effect of testicular sperm on clinical outcome of blastocyst transfer cycles.

To optimize embryo development and to increase the number of good quality embryos available for transfer, the culture system has been studied and developed (Swain et al., 2016). Good quality embryos that could be represented as implantation and livebirth should be regularly reviewed in clinical practice for quality control of IVF-ET. The success of ART, defined as delivery of a healthy child, depend on quality of the embryo culture environment. There are several key components of culture system such as air quality, culture media, type of incubator, pH (carbon dioxide), gas phase (5% or 20% oxygen concentration), temperature, medium storage, number of incubators, number of cultured embryos per drop and quality control. Specifically, conditions within the laboratory incubator, such as oxygen tension, pH, and temperature stability, could be the most important factors in embryo development as shown in many previous studied (Gardner et al., 2012; Swain, 2015; Higdon et al., 2008; Karagenc et al., 2004; Scott et al., 1993). In the IVF laboratory, gametes and embryos are cultured in sodium bicarbonate buffered media which maintains optimal pH under the 5-6% carbon dioxide and atmospheric/physiological oxygen concentrations. Previous studies have reported that the concentration of oxygen in the oviducts and uterus of most mammalian species is only 2%–8% (Mastroiannil and Jones, 1965; Fischer and Bavister, 1993; Ottosen et al., 2006). When gametes expose to high oxygen concentration as much as atmospheric oxygen concentrations (20% O_2), the gametes and embryos can be undergone under oxidative stress mediated by free oxygen radicals (Fischer and Bavister, 1993). Although Karagenc et al. (2004) has concluded that 20% oxygen concentration did not have effect on mouse embryo development up to blastocyst stages (Karagenc et al., 2004; Bahçeci et al., 2005). But, recent clinical data supported that more physiological condition, low oxygen concentration (5% O_2), could increase both implantation and live birth rate (Meintjes et al., 2009; Waldenström et al., 2009; Nanassy et al., 2010). Low oxygen culture condition can be set with tri-gas incubators and these incubators have used in many human IVF centers. However, the question remains, does the 20% oxygen concentration adversely effect on development of preimplantation embryos.

Multiple incubator types exist with varying capabilities and different methods of regulating their internal environment (Higdon et al., 2008; Swain, 2014). Standard large box-type incubators, initially developed to hold multiple flasks of somatic cells, have been long used for clinical IVF (Ham and Puck, 1962). They used thermal conductivity (TC) CO_2 sensor which are impacted by temperature and humidity. The humidity is provided via evaporation from a pan of water placed on the bottom of the incubator. But, the presence of a water pan is a potential source of microbial contamination, which can negatively affect the embryo development. Recently, a variety of moisture-free benchtop incubators outfitted with infrared (IR) sensors have been specifically developed for embryo cultivation (Swain, 2014). They provide a faster CO_2 recovery time than the incubator with TC CO_2 sensor following door opening. The moisture-free benchtop incubator consists of several small chambers with the heated surface which allow contact with the culture dish directly for fast temperature recovery, too (Fujiwara et al., 2007). These fast CO_2 and temperature recovery may be a benefit to culture condition of oocytes and embryos.

Several ARTs, such as, ICSI, TESE, blastocyst culture, embryo culture environment, and types of incubator were mentioned above affect embryo quality and pregnancy. The field of ART is extremely wide, and various factors can be impact to embryo quality and outcomes. Here, factors influencing on embryo quality and outcomes in the field of ART will be characterized.

Chapter 2

Assessing Comparative Efficiency of Intracytoplasmic Sperm Injection

2-1. INTRODUCTION

Intracytoplasmic sperm injection (ICSI) was initially developed to treat male factor infertility and has significantly improved fertilization rates (Palermo et al., 1992). ICSI has the potential to overcome possible fertilization problems when semen characteristics are poor or when fertilization rates in previous in vitro fertilization (IVF) cycles have been low (Bhattacharya et al., 2001). Although ICSI is indicated when a male factor for infertility exists, its application has been expanded to the cases of unexplained infertility, poor fertilization and low number of retrieved oocytes without comprehensible evidence to support its use (Fishel et al., 2000).

Fertilization failure would be the most distressful experience to both couples undergoing in-vitro fertilization and embryo transfer (IVF-ET) cycles and clinicians. Fertilization failure occurs in 3.52 ~ 20% of IVF cycles (Combelles et al., 2010; Ming et al., 2012) and even in 1~3% of ICSI cycles (Flaherty et al., 1998; Esfandiari et al., 2005). In ICSI cycles, rescue ICSI can also be performed when immature oocytes are matured one day after oocyte retrieval. Recently, however, concern over fertilization failure might cause the increase of the ICSI application to non-male factor infertility, especially in cycles that a small number of oocytes are retrieved, such as poor responders, and ovarian failure patients. Among non-male factor infertility, indications that ICSI is generally applied include unexplained infertility, retrieval of poor quality oocytes or a small number of oocytes, advanced maternal age, previous fertilization failure with conventional insemination, preimplantation genetic diagnosis (PGD), fertilization after in vitro maturation (IVM) and fertilization of frozen-thawed oocytes (Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2012). The main reason that ICSI is applied to these indications is to prevent the fertilization failure. According to the study of practical committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, ICSI is safe and effective for patients with male factor infertility and ICSI might benefit the patients undergoing IVF cycles with PGD, in-vitro matured oocytes and frozen-thawed oocytes. However, ICSI cannot improve clinical outcomes of patients with unexplained infertility, low oocyte yield and old age and there are no evidences that ICSI increase clinical outcomes in non-male factor infertility (Bhattacharya et al., 2001; Check et al., 2011). And also, it is unknown whether the reproductive risks known to be associated with ICSI in male factor infertility, such as increase of sex or autosomal chromosome aberrations, congenital anomalies and imprinting disorders, are associated with ICSI in non-male factor infertility (Practice Committee of the American Society of Reproductive Medicine, 2008). Routine use of ICSI for preventing fertilization failure may decrease the incidence of fertilization failure, but unnecessary ICSI has to be carried out in more than 30 couples to prevent one fertilization failure (Practice

Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2012).

On the other hand, despite the development of assisted reproductive technologies, prediction of fertilization failure or low fertilization with perfect accuracy is impossible in IVF cycles of patients seeking infertility clinic. Fertilization failure occurs unexpectedly after conventional insemination although husbands have normal sperm parameters. So, ICSI is usually suggested in the subsequent cycles when low fertilization or fertilization failure has occurred, although low fertilization or fertilization failure do not always occur repeatedly (Van et al., 2005; Kinzer et al., 2008). ICSI is usually applied in every subsequent cycle of the couple thereafter once ICSI is applied, either. It is not easy to choose one between conventional IVF and ICSI to fertilize the retrieved oocytes because there is possibility of fertilization failure in conventional IVF and there is possibility of unnecessary implementation in ICSI. Therefore, the implementation of split insemination has increased recently to minimize fertilization failure and check the possibility of fertilization by IVF, especially in the first IVF cycle of couples with unexplained infertility (Quaas and Dokras, 2008). Sibling oocytes are randomly allocated to conventional IVF or ICSI in split insemination. Fertilization results after split insemination will be helpful for determining insemination method, IVF or ICSI, in the subsequent cycles. Split insemination was mainly implemented in patients with unexplained infertility or mild male factor infertility and clinical outcomes of IVF and ICSI was compared in split insemination cycles (Khamsi et al, 2001; Hershlag et al., 2002, Plachot et al., 2002; Kihaile et al, 2003; Van et al, 2006; Fan et al., 2012; Johnson et al, 2013; Kim et al., 2015). However, effectiveness of split insemination is controversial. Fertilization failure could be prevented in some IVF cycles by ICSI but could not improve pregnancy outcome. Split insemination could not decrease significantly the implementation of ICSI in subsequent IVF cycles (Plachot et al, 2002). And also, there are concerns about the safety of ICSI in non-male factor infertility yet (Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2012).

Recently, application of ICSI is greatly extended into treatments of non-male factor infertility although its effectiveness is not proved in these cases. On the contrary, there are great concerns over the implementation of unnecessary ICSI in the treatment of non-male factor infertility. Main cause of ICSI application in non-male factor infertility is fear of the fertilization failure or low fertilization. It is difficult to compare the effect of insemination methods (IVF or ICSI) on fertilization of oocytes in separate IVF or ICSI cycles because differences among infertile couples might influence the fertilization of oocytes. Therefore, I compared the effect of insemination methods (IVF or ICSI) on fertilization in split insemination cycles to minimize the impact of these differences. I assessed the maturation statuses of unfertilized oocytes among inseminated oocytes to analyze whether other factor might influence the fertilization of oocytes. And also, I evaluated whether or not split insemination might contribute the increase of ICSI implementation by analyzing the rate of ICSI implementation in the second cycles of couples that pregnancy was not established in their first cycle.

2-2. MATERIALS AND METHODS

1. Patients, controlled ovarian hyperstimulation and fertilization

In this study, data was obtained from 571 IVF-ICSI split insemination cycles, 555couples (Table 2-1), and analyzed (Fig. 2-1). Among them, IVF cycles of 512 couples were first cycle. Couples of this study had tubal factor infertility (112 cycles), unexplained infertility (259 cycles), and mild male factor infertility (mild asthenozoospermia, 200 cycles). Females aged 40 and older were excluded from this study and cycles that more than 10 oocytes were retrieved were analyzed in this study. Fresh ejaculated sperms were used for fertilization. Patients whose sperm motility were less than 50% motility in the results of semen analysis were included in mild factor infertility. In several cycles, there were differences in sperm concentration and motility between the semen collected on the day of semen analysis and the semen collected on the day of oocyte pick-up. Clinicians explained that half of the retrieved oocytes were fertilized by conventional IVF and the other half were fertilized by ICSI. All couples submitted informed consent.

Controlled ovarian hyper-stimulation was carried out using GnRH antagonist, recombinant FSH or hMG, and hCG. Oocytes were retrieved transvaginally under ultrasound guidance at 35 hours after hCG injection. After completion of oocyte retrieval, the retrieved cumulus-oocyte complexes were randomly allocated into either conventional IVF or ICSI. The oocytes allocated to IVF were cultured in fertilization medium till sperm samples were prepared. The oocytes allocated to ICSI were prepared for ICSI. Cumulus cells were removed approximately 2 hours after oocyte retrieval. Cumulus cells were removed after short exposure to medium containing 80 IU/ml hyaluronidase (SAGE, Trumbull, Conneticut, USA). The remaining cells were mechanically removed by gentle pipetting with glass capillaries. The maturation status of the cumulus cell-removed oocytes was evaluated using inverted microscope (Nikon, Tokyo, Japan). The oocytes were maintained in fertilization medium for at least 1 hour before ICSI. Semen samples were collected immediately after oocyte pick-up. Sperm concentration and motility was analyzed by light microscopy. Sperm was prepared by density gradient centrifugation method using SpermGrad (Vitrolife, Göteborg, Sweden). The oocytes allocated to IVF were inseminated with sperm within five hours after oocyte retrieval. The oocytes were placed in fertilization medium with 50,000 or more motile sperms/ml overnight. Among the oocytes allocated to ICSI, mature MII oocytes were injected with sperm.

2. Assessment of fertilization and embryo quality

Fertilization was assessed approximately from 16 to 18 hours after insemination or ICSI. Normal fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body. In all split insemination cycles, fertilization rate of IVF and fertilization rate of ICSI were calculated independently. Fertilization rate of conventional IVF was represented as the percentage of fertilized oocytes per inseminated (or retrieved) oocytes. In ICSI, two fertilization rates were calculated; the percentage of fertilized oocytes per injected oocytes and the percentage of fertilized oocytes per retrieved oocytes (oocytes that were allocated to ICSI). After the fertilization statuses of oocytes was observed, the fertilized oocytes were cultured in a 50μl drop of medium covered with paraffin oil and in humidified atmosphere under 6% CO2 at 37℃ before embryo replacement. Embryos fertilized by conventional IVF and embryos fertilized by ICSI were cultured separately. Embryos were replaced at between day 2 and day 5. Embryo quality was assessed just before embryo replacement. Embryo quality was classified according to the embryo morphology at day of embryo replacement. Comparison of quality between embryos from conventional IVF and embryos from ICSI was performed only in cycles that embryos transfer was performed at day 3 after oocyte retrieval. Day 3 embryo transfers were performed in 301 cycles. Embryos were classified into good, fair or poor embryos based on their morphologies (fig. 1-2). Good embryos had ≥ 7 even-sized blasotomeres and no or ≤ 10% fragmentation of the volume of the embryos. Fair embryos had ≤ 6 even-sized or ≥ 6 uneven-sized blastomeres and 10 ~ 30% fragmentation. Poor embryos had uneven-sized blastomeres, regardless of blastomere numbers and > 30% fragmentation. Embryos from conventional IVF were replaced in 166 cycles, embryos from ICSI were replaced in 66 cycles and embryos from conventional IVF were replaced along with embryos from ICSI in 278 cycles. Clinical pregnancy was ascertained by confirming fetal heart beat using ultrasonography at six or seven weeks of gestation.

3. Statistical analysis

Results are expressed as mean±SD. Statistical analysis was performed using the SPSS 12.0 for Windows software package. One-way analysis of variance (ANOVA), the Kruskal-Wallis rank test, or Fisher's exact test was performed to analyze the significance of differences between groups. A P value of < 0.05 was considered to indicate a statistically significant difference among groups.

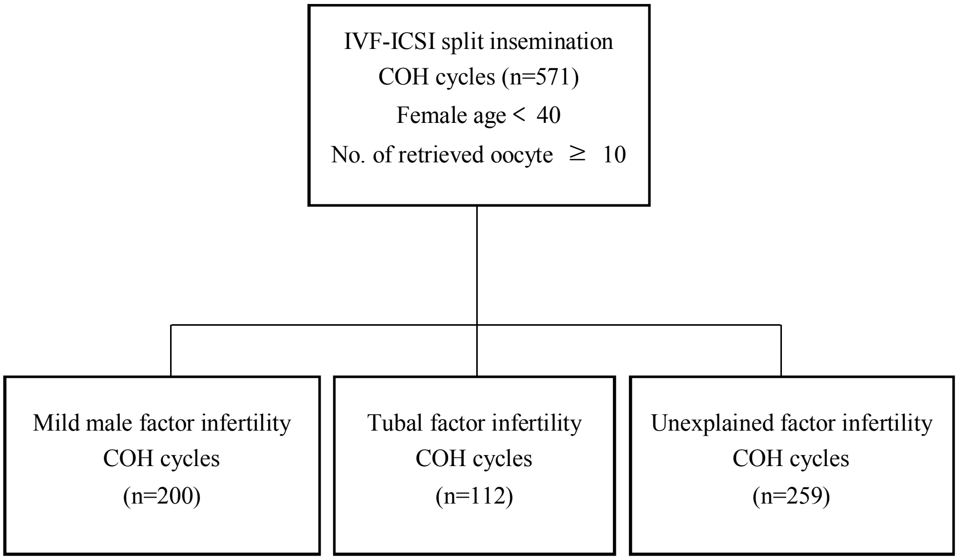

Figure 2-1. A schematic diagram for patient selection to evaluate the effect of insemination methods (conventional IVF or ICSI) on mild or non-male factor.

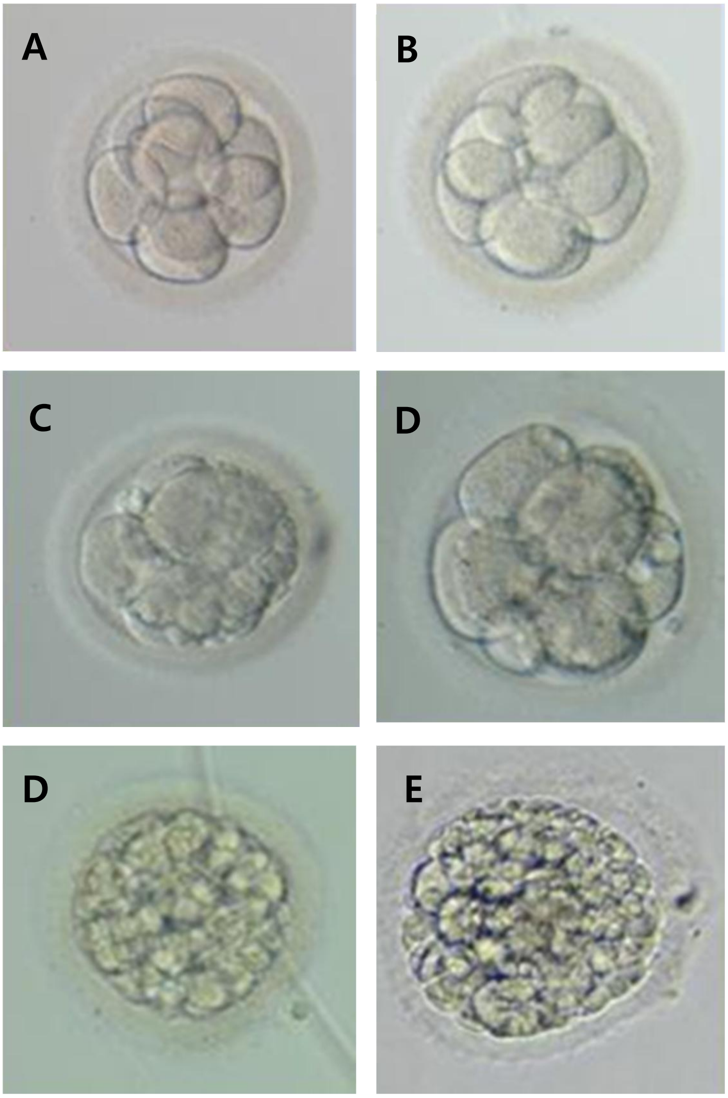

Figure 2-2. Photomicrographs of day 3 embryo with different quality. A, B. good grade embryos had ≥ 7 even-sized blastomeres and no or ≤ 10% fragmentation of the volume of the embryos; C, D. fair grade embryos had ≤ 6 even-sized or ≥ 6 uneven-sized blastomeres and 10 ~ 30% fragmentation; E, F. poor grade embryos had uneven-sized blastomeres, regardless of blastomere numbers and > 30% fragmentation.

2-3. RESULTS

1. Comparison of fertilization rates between conventional IVF and ICSI in IVF-ICSI split insemination cycles

A total of 10,471 oocytes were retrieved in 571 cycles. Among them, 4,915 oocytes were allocated to IVF and 5,556 oocytes were allocated to ICSI. In IVF, normal fertilization (2PN and second polar body) was observed in 2,878 oocytes (58.6%). In ICSI, 3,926 oocytes were injected with sperm and normal fertilization was observed in 2,854 oocytes (51.4%). Fertilization rates of conventional IVF and ICSI were compared in IVF-ICSI split insemination cycles (Table 2-2). Fertilization rate per injected oocytes of ICSI (72.3±24.3%) was significantly higher ($P<0.001$) than fertilization rate per inseminated (or retrieved) oocytes of conventional IVF (59.2±25.9%). However, fertilization rate per retrieved oocytes of ICSI (52.1±22.5%) was significantly lower ($P<0.001$) than fertilization rate per inseminated (or retrieved) oocytes of conventional IVF (59.2±25.9%).

Total fertilization failure and low fertilization (fertilization rate < 30%) were compared between conventional IVF and ICSI in split insemination cycles (Table 2-3). Total fertilization failure was occurred in 23 cycles of conventional IVF (4.0%) and in eight cycles of ICSI (1.4%) among 571 cycles. Total fertilization failure was occurred significantly higher ($P<0.05$) in conventional IVF than in ICSI. Low fertilization rate per inseminated oocytes of conventional IVF was 11.4% (65/571) and low fertilization rate per injected oocytes of ICSI was 7.0% (32/571). Low fertilization rate was significantly higher ($P<0.05$) in conventional IVF than in ICSI when fertilization rate was calculated per inseminated oocytes in IVF and it was calculated per injected oocytes in ICSI. However, low fertilization rate per retrieved oocytes of conventional IVF (11.4%) was significantly lower ($P<0.05$) than that of ICSI (17.2%).

Table 2-1. Comparison of patient characteristics among couples with three different infertility factors

	Total	Mild male factor	Tubal factor	Unexplained factor	P-value
No. of cycles	571	200	112	259	
Female age	34.0±2.9	34.1±2.8 [a]	33.0±3.3 [b]	34.3±2.8 [a]	0.000
FSH level (mIU/ml)	6.7±1.9	6.6±1.8	6.4±2.0	6.9±1.9	0.075
E2 level (pg/ml)	3,670.6 ± 1,748.2	3,876.1 ± 1,990.2	3,921.9 ± 1,947.2	3,533.9 ± 1,588.0	0.334
Sperm concentration before treatment (x10^6/ml)	108.8 ± 66.5	104.5 ± 72.9	96.4 ± 56.2 [c]	117.6 ± 30.5 [d]	0.0096
Sperm motility before treatment (%)	57.3 ± 26.7 %	41.4 ± 20.2 % [e]	61.1 ± 17.0 % [f]	50.9 ± 11.7 % [f]	0.0000
Sperm concentration after treatment (x10^6/ml)	29.4 ± 11.9	26.1 ± 12.1 [e]	29.5 ± 11.9	32.0 ± 11.2 [f]	0.0000
Sperm motility after treatment (%)	90.8 ± 8.6 %	88.0 ± 10.7 % [e]	92.6 ± 5.1 % [f]	92.1 ± 7.3 % [f]	0.0000

Values are represented as mean ± SD

[a,b] significantly different from each other in the same column ($P<0.01$)

[c,d] significantly different from each other in the same column ($P<0.05$)

[e,f] significantly different from each other in the same column ($P<0.0001$)

Table 2-2. Comparison of fertilization rate between IVF and ICSI in split insemination cycles

Indication of infertility	Total	Mild male factor	Tubal factor	Unexplained factor
No. of cycles	571	200	112	259
No. of retrieved oocytes	18.3±7.9	18.4±7.6	18.5±7.0	18.2±7.8
Fertilization rate of IVF * (per inseminated oocytes)	59.2 ± 25.9 % [a]	59.5 ± 26.4 % [a]	59.3 ± 27.7 % [a]	58.9 ± 24.9 % [a]
Fertilization rate of ICSI * (per injected oocytes)	72.3 ± 24.3 % [b]	72.5 ± 23.9 % [b]	72.2 ± 25.1 % [b]	72.1 ± 24.4 % [b]
Fertilization rate of ICSI * (per retrieved oocytes)	52.1 ± 22.5 % [c]	53.6 ± 22.6 %	53.6 ± 23.0 %	50.3 ± 22.1 % [c]
No. of embryo transfer cycles	510	183	98	229
No. of transferred embryos	3.0±0.8	3.0±0.7	3.1±0.8	3.0±0.9
No. of pregnancy cycles (%)	243 (47.6)	89 (48.6)	51 (52.0)	103 (45.0)

* Values are represented as mean ± SD

[a,b]; [a,c] significantly different from each other (P<0.001)

2. Comparison of fertilization rates between conventional IVF and ICSI in couples with three different infertility factors

Fertilization rates of conventional IVF and ICSI were compared in couples with three different infertility factors; mild asthenozoospermia, tubal factor and unexplained factor. Females were significantly younger ($P<0.001$) in couples with tubal factor (33.0 ± 3.3) than in couples with mild male factor or unexplained factor (34.1 ± 2.8, 34.3 ± 2.8, respectively). Number of retrieved oocytes, follicle-stimulating hormone (FSH) level at day 2 or 3 of menstrual cycle, E2 level at the day before oocyte retrieval were not different among couples with three different infertility factors (Table 2-1). Clinical pregnancy rate was not different among these couples (Table 2-2). Sperm concentration and motility before and after preparation for IVF were compared (Table 2-1). There are differences in sperm concentration and motility before sperm preparation for IVF among these couples. Sperm concentration of couples with tubal factor ($95.4 \pm 56.5 \times 10^6$/ml) was significantly lower ($P<0.05$) than that of couples with unexplained factor ($117.6 \pm 30.5 \times 10^6$/ml). Sperm concentration of couples with mild asthenozoospermia ($104.5 \pm 72.9 \times 10^6$/ml) was not different compared to those of couples with other infertility factor. Sperm motility of couples with mild asthenozoospermia (41.4 ± 20.2%) was significantly lower ($P<0.0001$) than those of couples with tubal factor or unexplained factor (61.1 ± 17.0% and 50.9 ± 11.7%, respectively). There were differences in sperm concentration and motility after sperm preparation for IVF using discontinuous density gradient method among couples with three different infertility factors. Sperm concentration of couples with mild asthenozoospermia (26.1 ± 12.1 x 10^6/ml) was significantly lower ($P<0.0001$) than that of couples with unexplained factor (32.0 ± 11.2 x 10^6/ml). Sperm concentration of couples with tubal factor (29.5 ± 11.9 x 10^6/ml) was not different compared with those of couples with mild asthenozoospermia or unexplained factor. Sperm motility of couples with mild asthenozoospermia (88.0 ± 10.7%) was significantly lower ($P<0.0001$) than those of couples with tubal factor or unexplained factor (92.6 ± 5.4% and 92.1 ± 7.3%, respectively).

Fertilization rate per injected oocytes of ICSI was significantly higher ($P<0.001$) than fertilization rate per inseminated oocytes of conventional IVF in all couples with three different infertility factors. However, fertilization rate per retrieved oocytes of ICSI was lower than fertilization rate per inseminated oocytes of conventional IVF (Table 2-2). In couples with mild asthenozoospermia, total fertilization failure was significantly higher ($P<0.05$) in conventional IVF (5.0%) than in ICSI (1.0%) and low fertilization was not different between conventional IVF and ICSI. In couple with tubal factor, total fertilization and low fertilization were not different between conventional IVF and ICSI. In couples with unexplained factor, total fertilization failure was not different between conventional IVF and ICSI but low fertilization rate per inseminated oocytes of conventional IVF (11.6%)

was significantly higher (P<0.05) than low fertilization rate per injected oocytes of ICSI (5.8%) (Table 2-3).

3. Comparison of embryo quality between conventional IVF and ICSI in split insemination cycles and pregnancy outcomes depending on transferred embryos' origin

Qualities of embryos fertilized by conventional IVF or ICSI were compared in total of 301 cycles that embryos were transferred at day 3 (Table 2-4). The rate of good quality embryos was not different between embryos from conventional IVF (16.6 ± 23.2%) and embryos from ICSI (16.6 ± 26.6%). The rate of fair quality embryos was significantly higher (P<0.05) in embryos from ICSI (53.0 ± 34.8%) than in embryos from conventional IVF (47.7 ± 29.4%). And the rate of poor quality embryos was significantly higher (P<0.05) in embryos from conventional IVF (35.6 ± 29.6%) than in embryos from ICSI (29.8 ± 33.4%).

All embryos were frozen in 59 cycles, embryo replacements were cancelled due to the poor qualities of embryos in 2 cycles and embryos were replaced in 510 cycles. Positive β-hCG was confirmed in 276 cycles and 31 cycles ended in biochemical pregnancies. Of 245 clinical pregnancies, eight pregnancies were ectopic pregnancies, 21 pregnancies were aborted within the first trimester of gestation, 4 pregnancies were terminated, 4 pregnancies were lost with the second trimester of gestation, and 168 pregnancies resulted in live-births. Main cause of termination was incompetent internal os of cervix (IIOC). Follow-ups of 40 pregnancies were failed because contact with the patients was lost after clinical pregnancies were confirmed. Pregnancy outcomes were analyzed according to origin of transferred embryos (Table 2-5). Only embryos from conventional IVF were replaced in 166 cycles. Among the cycles, fertilization failure has occurred in 4 cycles of ICSI and all fertilized oocytes from ICSI were frozen in 64 cycles. In these cycles, mean age of females was 33.6 ± 2.6 years old and mean number of replaced embryos was 2.8±0.2. Clinical pregnancies were confirmed in 80 cycles (48.2%). In 66 cycles, only embryos from ICSI were replaced. Among the cycles, fertilization failure has occurred in 17 cycles of IVF and all fertilized oocytes from IVF were frozen in one cycle. Mean age of females was 33.8 ± 2.8 years old and mean number of replaced embryos was 2.8 ± 0.8. Clinical pregnancies were confirmed in 33 cycles (50.0%). Embryos from conventional IVF were replaced along with embryos from ICSI in the remaining 278 cycles and clinical pregnancies were confirmed in 132 cycles (47.5%) among them. Mean age of female was 34.4 ± 3.1 years old and mean number of replaced embryos was 3.3 ± 0.7 in these cycles. Female age was significantly higher ($P<0.05$) in patients that embryos from IVF were transferred along with embryos from ICSI than in patients that only embryos from IVF were transferred. And significantly more ($P<0.05$) embryos were cultured in patients that only embryos from IVF were transferred (8.5 ± 2.4) than in patients that embryos from IVF were transferred along with embryos from ICSI (7.9 ± 2.2) or in patients that only embryos from ICSI were transferred (7.3 ± 2.9). Significantly more ($P<0.001$) embryos were transferred in patients that embryos from IVF were transferred along with embryos from ICSI (3.3 ± 0.7) than in patients that only embryos from ICSI were transferred (2.8 ± 0.8) or in patients that only embryos from IVF were transferred (2.8 ± 0.2). However, clinical pregnancy, abortion, ectopic pregnancy, termination, second trimester loss and live-birth were not different among three groups ($P=0.933$).

Table 2-3. Comparison of total fertilization failure and low fertilization between IVF and ICSI in split insemination cycles

Indication of infertility	No. of cycles	IVF		ICSI		
		Total fertilization failure	Low fertilization (per retrieved oocytes)	Total fertilization failure	Low fertilization (per injected oocytes)	Low fertilization (per retrieved oocytes)
Mild male factor	200	10 (5.0 %) [a]	21 (10.5 %)	2 (1.0 %) [b]	11 (5.5 %)	30 (15.0 %)
Tubal factor	112	5 (4.5 %)	14 (12.5 %)	3 (2.7 %)	6 (5.4 %)	14 (12.5 %)
Unexplained factor	259	8 (3.1 %)	30 (11.6 %) [h]	3 (1.2 %)	15 (5.8 %) [i]	46 (17.8 %)

[a,b]; [c,d]; [c,e] significantly different from each other ($P<0.05$)

Table 2-4. Comparison of embryo quality between IVF and ICSI in split insemination cycles

Indication of infertility	No. of cycles	Embryo quality (%)					
		IVF			ICSI		
		Good	Fair	Poor	Good	Fair	Poor
Total	301	16.6±23.2	47.7±29.4 [a]	35.6±29.6 [c]	16.6±26.6	53.0±34.8 [b]	29.8±33.4 [d]
Mild male factor	108	16.9±22.2	47.9±30.5	35.1±29.8	17.8±26.3	53.5±34.3	28.9±33.0
Tubal factor	59	21.0±28.1	48.1±30.7	30.9±29.3	16.3±25.3	54.4±34.8	27.2±31.8
Unexplained factor	134	14.5±21.5	47.4±28.1	38.0±29.6	15.8±27.6	52.1±35.5	31.6±34.6

Values are represented as mean ± SD

[a,b]; [c,d] significantly different from each other (*P*<0.05)

Table 2-5. Pregnancy outcomes according to the origin of transferred embryos in split insemination cycles

	Origin of transferred embryos				P-value
	IVF/ICSI	ICSI	IVF	Total	
No. of ET cycles	278	66	166	510	
Average female age *	34.4 ± 3.1 [a]	33.8 ± 2.8	33.6 ± 2.6 [b]	34.1 ± 2.9	0.033
Average no. of cultured embryos *	7.9 ± 2.2 [c]	7.3 ± 2.9	8.5 ± 2.4 [d]	8.0 ± 2.4	0.001
Average no. of transferred embryos *	3.3 ± 0.7 [e]	2.8 ± 0.8 [f]	2.8 ± 0.2 [f]	3.1 ± 0.8	0.000
(+) β-hCG (%) **	155 (55.8)	35 (53)	91 (54.8)	276 (54.1)	0.920 ****
Clinical pregnancy (%) **	132 (47.5)	33 (50.0)	80 (48.2)	245 (48.0)	0.933 ****
Ectopic pregnancy (%) ***	4 (3.0)		4 (5.0)	8 (3.3)	0.387 *****
Clinical abortion (%) ***	10 (7.6)	3 (9.1)	8 (10.0)	21 (8.6)	0.824 *****
2nd trimester loss (%) ***	3 (2.3)	1 (3.0)	1 (1.3)	4 (1.6)	0.620 *****
Termination (%) ***	2 (1.5)	1 (3.0)	1 (1.3)	4 (1.6)	0.784 *****
Follow-up loss (%) ***	23 (17.4)	3 (9.1)	14 (17.5)	40 (16.3)	0.481 *****
Livebirth (%) ***	90 (32.4)	26 (39.4)	52 (31.3)	168 (32.9)	0.353 *****

* Values are represented as mean ± SD; ** percentage was calculated from total cycles of respective group; *** percentage was calculated from clinical pregnancies of respective group; **** P-value was calculated from total cycles of respective group; ***** P-value was calculated from total clinical pregnancies of respective group

[a,b]; [c,d] significantly different from each other (*P*<0.05)

[e,f] significantly different from each other (*P*<0.0001)

Table 2-6. Insemination methods of subsequent cycles and fertilization rate of respective insemination methods of the first cycle

Insemination method of subsequent cycles	Fertilization rate of respective insemination method of the first cycles				Total
	IVF ≥ 50% ICSI ≥ 50%	IVF ≥ 50% ICSI < 50%	IVF < 50% ICSI ≥ 50%	IVF < 50% ICSI < 50%	
IVF (%)	20 (21.1%)	18 (18.9%)		8 (8.4%)	46 (48.4%)
IVF+ICSI (%)	7 (7.4%)	7 (7.4%)	8 (8.4%)	5 (5.3%)	27 (28.4%)
ICSI (%)	3 (3.2%)	2 (2.1%)	10 (10.5%)	7 (7.4%)	22 (23.2%)
Total (%)	30 (31.6%)	27 (28.4%)	18 (18.9%)	20 (21.1%)	95

4. Contribution of IVF-ICSI split insemination to increase of ICSI cycles

IVF-ICSI split insemination was undergone in 571 cycles of 555 couples. Among them, the IVF cycles of 521 couples were their first cycles. Ninety-five couples were not pregnant in their first cycle and underwent the second IVF cycle. Insemination techniques were analyzed in the second cycles; conventional IVF, ICSI or split insemination (Table 2-6). Insemination method of the second cycle was analyzed depending on the fertilization rate of conventional IVF and ICSI of their first cycles. In 30 of the 95 second cycles, fertilization rates of conventional IVF and ICSI of the first cycles were over 50%. Among them, conventional IVF was carried out in 20 cycles, split insemination in seven cycles and ICSI in three cycles. In 27 of the second cycles, fertilization rate of conventional IVF of the first cycle was over 50% and fertilization rate of ICSI of the first cycle was under 50%. Among them, conventional IVF was carried out in 18 cycles, split insemination in seven cycles and ICSI in two cycles. In 18 of the second cycles, fertilization rate of conventional IVF of the first cycle was under 50% and fertilization rate of ICSI of the first cycle was over 50%. Conventional IVF was not carried out in second cycles, split insemination in eight cycles and ICSI in 10 cycles. In 20 of the second cycles, both fertilization rates of conventional IVF and ICSI of the first split insemination cycles was under 50%. Conventional IVF was carried out in eight cycles, split insemination in five cycles and ICSI in seven cycles. Split insemination or ICSI was carried out in nineteen cycles although the fertilization rate of conventional IVF was over 50% in their first cycle. Sperm concentration and motility (93.1 ± 41.4 x 10^6/ml, 56.5±19.3%) were not changed in their second cycles compared with those of their first cycles (102.5 ± 61.1 x 10^6/ml, 47.9 ± 15.4%). ICSI should be carried out in one cycle since sperm concentration and motility of the second cycle (6.5 x 10^6/ml, 19.0%) got worse compared with those of the first cycles (24.3 x 10^6/ml, 32.9%).

2-4. DISCUSSION

In split insemination cycles, fertilization rate is somewhat different between IVF and ICSI depending on studies. In many studies, fertilization rate of ICSI was higher than that of IVF when fertilization rates of them were calculated from the allocated oocytes to IVF or ICSI (Kim et al., 2015; Jaroudi et al., 2003; Hwang et al., 2005; Yoeli et al., 2008). However, it has been reported that the fertilization rate of IVF is similar to or higher than that of ICSI in several studies (Bhattacharya et al., 2001; Fan et al., 2012; Ming et al., 2015). In the present study, fertilization rate was significantly higher in IVF than in ICSI when fertilization rate was calculated from allocated oocytes. I observed the maturation statuses of unfertilized oocytes among inseminated oocytes when the fertilization statuses of them were checked the next morning after oocyte retrieval. Four thousand nine hundred fifteen of 10,471oocytes are allocated to IVF. Among them, 2,878 oocytes were normally fertilized (2PN and 2PB, 58.6%) and 392 oocytes were abnormally fertilized (1PN or ≥ 3PN, 8.0%). Seven hundred seventy one oocytes were metaphase II oocytes (15.7%), 749 oocytes were immature oocytes (MI or GV stage, 15.2%) and 125 oocytes were abnormal or degenerated oocytes (2.5%). Immature, abnormal or degenerated oocytes are oocytes that cannot be fertilized. Some of metaphase II oocytes would be immature oocyte at the time of oocyte retrieval and underwent maturation between oocyte retrieval and observation of fertilization status (about 1 day) and the others would be mature oocyte at the time of oocyte retrieval but were not fertilized.

On the contrary, some of the fertilized oocytes would be immature oocytes at the time of oocyte retrieval but underwent maturation and be fertilized between oocyte retrieval and observation of fertilization status. According to these results, most of mature oocytes at the time of oocyte retrieval would be fertilized and very a few oocytes would not be fertilized. The practice committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology have reported that fertilization failure seems to correlate with poor ovarian stimulation (Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2012). Fertilization after IVF also seems to correlate with ovarian stimulation. Therefore, fertilization rate after IVF seems to be improved if ovarian stimulation is carried out in a way that increases the rate of mature oocytes among retrieved oocytes although fertilization of oocytes is influenced by many factors.

Similar to recent studies, fertilization failure occurred more frequently in IVF than in ICSI in the present study. Low fertilization of which rate was less than 30% was also higher in IVF than ICSI when fertilization rate of ICSI was calculated from the injected oocytes. However, low fertilization was significantly higher in ICSI than in IVF when it was calculated from allocated oocytes. Low fertilization depending on infertility indication was also similar between IVF and ICSI or was higher in ICSI than in IVF when fertilization rate was calculated from allocated oocytes although the difference was not significant. Fertilization failure was significantly higher in only mild male factor infertility when fertilization failure was analyzed based on infertility indication. According to these results, it seems that ICSI is not beneficial in terms of fertilization compared with IVF. However, ICSI may decrease the incidence of fertilization failure in some infertility indications, such as mild male factor, although ICSI has to be implemented unnecessarily.

Good quality embryo rates were not different between IVF and ICSI. This result is consistent with the results of others (Yoeli et al., 2008; Staessen et al., 1999; Verheyen et al., 1999; Bukulmez et al., 2000; Fishel et al., Taylor et al., 2008). However, fair quality embryo rate was significantly higher in ICSI than in IVF and poor quality embryo rate was significantly higher in IVF than in ICSI. Overall, quality of embryos was better in ICSI than in IVF. The reason for this is likely to that some of IVF embryos were derived from immature oocytes. Namely, some immature oocytes at the time of oocyte retrieval underwent maturation and was fertilized during IVF and the embryos derived from theses oocytes were mixed in the embryos derived from in vivo matured oocytes. On the contrary, all ICSI embryos are derived from oocytes that underwent maturation before ICSI was implemented. For this reason, it seems that the overall quality of ICSI embryos was better than that of IVF embryo. It is difficult to evaluate the developmental potential of embryos derived from immature oocytes matured during IVF. According to the clinical outcomes of ICSI, developmental potential of embryos derived from in vitro matured oocytes is inferior to that of embryos derived from in vivo matured oocytes.

Cleavage rate of oocytes fertilized from in vitro matured oocyte was lower than that of fertilized oocytes of which maturation stage were metaphase II at denudation (Balakier et al., 2004; Strassburger et al., 2004; Shu et al., 2007). And good quality embryo rate was lower in embryos derived from in vitro matured oocytes than in embryos derived from mature oocytes (Ko et al., 2015). Pregnancy outcomes were not different among cycles depending on the origin of replaced embryos; cycles that only IVF embryos were replaced, cycles that only ICSI embryos were replaced and cycles that IVF embryos were replaced together with ICSI embryos.

There are limitations in my study. Selection of transferred embryos could not be controlled due to the retrospective design of this study. Embryos with good quality are preferentially selected for embryo transfer. And embryos from IVF are preferentially selected over the embryos from ICSI when embryo qualities are similar between them. Therefore, there are limitations in comparison of pregnancy rates among study groups. I have analyzed as many split insemination cycles as possible to overcome such limitations. And also I have limited the age of female (< 40 years old), the number of retrieved oocytes (≥ 10) and the infertility factors of patients. However, prospective randomized study is needed on a large scale to analyze whether the split insemination is effective. In conclusion, fertilization rate and embryo quality were not different between IVF and ICSI in split insemination cycles. Total fertilization failure was significantly lower in ICSI than in IVF. However, fertilization rate of IVF were similar to or higher than that of ICSI in

78.9 % of the split insemination cycles (451/571 cycles). Unnecessary ICSI has been implemented in these cycles. Therefore, the implementation of split insemination should be considered carefully.

Chapter 3

Potency of Testicular Sperm to Support Embryonic Development to the Blastocyst Stage

3-1. INTRODUCTION

Male factor infertility has been overcome successfully and high pregnancy rates have been reported after treatment of male factor infertility by intracytoplasmic sperm injection (ICSI). The application of ICSI has gradually expanded since its introduction, and recently, it has been extensively applied not only in the treatment of male factor infertility, but also in the treatment of female factor infertility. Although ICSI is widely applied, it remains controversial whether the source or quality of sperm used to perform ICSI affect the clinical outcomes, such as fertilization, development to blastocyst, implantation and pregnancy.

The quality of sperm can be very different depending on the histopathological causes in men - even if the sperm are retrieved from same source. Because sperm qualities differ among men, the clinical outcomes of ICSI might be affected by sperm quality. A number of studies have reported that clinical outcomes of human in vitro fertilization (IVF) were better when the quality of sperm used to fertilize retrieved oocytes was good, rather than when sperm quality was poor (Janny et al., 1994; Shoukir et al., 1998).

The sperm sources used for ICSI are various. ICSI can be performed using ejaculated sperm (EJ), epididymal sperm or testicular sperm (TE) (Craft et al., 1993; Palermo et al., 1992; Schoysman et al., 1993). There are many studies comparing the clinical outcomes of ICSI according to sperm sources. However, there is still controversy about the clinical outcomes of ICSI according to sperm sources. Several studies have reported the improved clinical ICSI outcomes when EJ or epididymal sperm were used for ICSI than when TE were used. Compared to EJ or epididymal sperm, fertilization rates were significantly lower when ICSI was performed using TE. Blastocyst formation rate was also lower when ICSI was performed using TE, than when ICSI was performed using EJ (Balaban et al., 2001; Rossi et al., 2003). Moreover, implantation rates were significantly higher and the rate of miscarriage was significantly lower in the latter than in the former. The number of embryos which developed to blastocyst stage by day five was the lowest in embryos originating from TE of men with azoospermia. Sperm retrieved from men with defective spermatogenesis have been expected to adversely affect the viability, quality, and development of embryos (Aziz and Agarwal, 2008; Lee et al., 2009; Nallella et al., 2006). The effect of sperm on embryonic development, namely paternal effect, has been described in a number of recent studies. It has been suggested that sperm retrieved from men with defective spermatogenesis will have a negative paternal effect on embryonic development. However, to date, a negative paternal effect of such sperm on embryonic development is unclear and controversial.

Many studies have reported that clinical outcomes of ICSI using TE are inferior to those of ICSI using EJ, but several studies have reported that clinical outcomes of ICSI were similar regardless of sperm sources (Greco et al., 2005; Weng et al., 2014). According to the studies, fertilization rate and embryonic development were not different between ICSI cycles using EJ and those using TE. And the ability of TE to support embryonic development is comparable to that of EJ or epididymal sperm. In single blastocyst transfer cycles, ongoing pregnancy rates did not differ between cycles in which epididymal or TE were used for ICSI and cycles in which EJ were used for ICSI. Furthermore, the rate was similar to that of IVF cycles in which conventional insemination was performed using EJ. And the rate of cycles in which one or more embryos developed to blastocysts did not differ among them. The implantation and pregnancy rates were not different when ICSI was performed using ejaculated, epididymal or testicular sperm and cleavage stage embryos were transferred on day three. There were also no significant differences in miscarriage rates, although the rate was slightly higher when ICSI was performed using TE than when EJ or epididymal sperm were used. In another study, implantation rates were significantly higher in cycles in which ICSI was performed using TE than in cycles where EJ or epididymal sperm were used (Braga et al., 2013; Naru et al., 2008; Nilsson et al., 2007; Xie et al., 2014).

To date, it remains unclear whether the quality or source of sperm can affect the clinical outcomes of ICSI. As to whether the ability of TE to support embryonic development to blastocyst stage is comparable to that of EJ is currently under debate. To evaluate whether the sperm source can affect the clinical outcomes of ICSI, the clinical outcomes were analyzed in cycles in which ICSI was performed using EJ or TE and embryos were transferred on day five. In addition, the ability of sperm to support embryonic development to blastocyst stage was compared between EJ and TE sperm by assessing blastocyst formation and quality in those cycles.

3-2. MATERIALS AND METHODS

1. Patients, controlled ovarian hyper-stimulation and fertilization

Data from 178 blastocyst transfer cycles of 172 couples underwent IVF-ET program (Fig. 3-1). All women's age was under 34 years old. ICSI was performed using ejaculated sperms in 141 cycles of 135 couples (EJ-group) and using testicular sperms in 37 cycles of 35 couples. Embryos were transferred at day 5 in all cycles. In EJ-group, indications of infertility were male factor infertility (90 cycles of 84 couples), female factor infertility (27 cycles of 27 couples) and unexplained infertility (9 cycles of 9 couples). Fifteen couples had female factor and male factor infertility (15 cycles). In TE-group, testicular sperm extraction (TESE) was performed due to non-obstructive azoospermia in 12 patients (10 cycles) and obstructive azoospermia in 25 patients (25 cycles). Among non-obstructive azoospermia patients, two were patients with retrograde ejaculation. TESE was performed in them because no sperm was present in the urine.

Controlled ovarian hyper-stimulation was carried out using GnRH agonist or GnRH antagonist, recombinant FSH or hMG and hCG. Oocytes were retrieved transvaginally under ultrasound guidance at 35 hours after hCG injection. Cumulus cells were removed enzymatically and then mechanically approximately 2 hours after oocyte retrieval. Cumulus cells were removed by brief exposure of cumulus-oocyte-complexes to medium containing 80IU/ml hayaluronidase (SAGE, Trumbull, Connecticut, USA). And then, cumulus cells that were not removed by exposing to hyaluronidase were removed completely by aspirating oocytes in and out hand-drawn glass capillaries. The status of oocyte maturation was observed under an inverted microscope (Nikon, Tokyo, Japan). ICSI was performed only in metaphase II oocytes using ejaculated or testicular sperms more than 1 hour after removal of cumulus cells.

2. Testicular sperm extraction

Physical examination, hormone profiling and semen analyses were performed. Men were diagnosed as azoospermia when no sperms were present in three consecutive semen analyses. Non-obstructive azoospermia was diagnosed based on the histopathologic results of testicular biopsy. TESE was performed on the day of or the day before oocyte retrieval. Small incision was made in scrotum and tunica vaginalis. Tunica albuginea was opened and a small piece of exposed testicular tissue was excised. The testicular tissue was gently dissected under a dissecting microscope after several rinses with Ham's F-10 medium supplemented with 0.4% human serum albumin. Seminiferous tubules were squeezed using two pairs of fine forceps after careful removal of blood vessels, connective and other tissues. The presence of spermatozoa was examined under a microscope. Testicular sperms were incubated at 37℃, 6% CO_2 in air until ICSI was performed.

3. Assessment of fertilization and embryo quality

Fertilization was assessed 16 hours after ICSI. Only oocytes with two pronuclei and the second polar body was assessed as normally fertilized oocytes. Some of the fertilized oocytes were frozen at pronucleus stage after the fertilization statuses of oocytes were assessed. The remaining fertilized oocytes were washed several times and transferred to cleavage medium. The zygotes were cultured to blastocyst stage in a 50μl medium droplets covered with paraffin oil and in humidified atmosphere at 37℃. Culture medium was changed from cleavage medium to blastocyst medium at day 3 after oocyte retrieval.

Embryo quality was assessed immediately before changing the culture medium at day 3 and before embryo transfer at day 5. At day 3, embryos were classified into good, fair and poor embryos according to the number of blastomeres, symmetry of blastomeres and the degree of fragmentation. Embryos with more than 7 even-sized blastomeres and less than 10% fragmentation were classified as good embryos. Embryos with more than 6 uneven-sized blastomeres and 11-40% fragmentation were classified as fair embryos. Embryos with more than 40% fragmentation, regardless of the blastomere number and symmetry, or embryos with less than 5 even or uneven blastomeres were classified as poor embryos. Blastocysts were classified into six grades based on the degree of expansion and the status of hatching (Gardner and Schoolcraft, 1999): early blastocyst (Grade 1), middle blastocyst (Grade 2), blastocyst (Grade 3), expanded blastocyst (Grade 4), hatching

(Grade 5) or hatched blastocyst (Grade 6). Subsequently, quality of inner cell mass (ICM) and trophectoderm (TE) were assessed in blastocysts except early and middle blastocysts. ICM was classified into three grades: ICM with many tightly packed cells (grade A), several loosely packed cells (grade B) or very few cells (Grade C). TE was also classified into three grades: TE with many cohesive cells (Grade A), several loose cells (Grade B) or very few large cells (Grade C). Fully expanded blastocysts (≥ grade 4) with grade A ICM and grade A or B TE were classified into good blastocysts. Blastocysts (≥ grade 3) with grade B ICM and grade A or B TE were classified into fair blastocysts. Early, middle blastocyst or blastocysts with grade C ICM and TE, regardless of the expansion degree and hatching status, were assigned into poor blastocysts (fig. 3-2, fig. 3-3). Embryo quality was compared between EJ and TE group.

4. Statistical analysis

Continuous data are expressed as mean±SD and categorical data are presented as percentage frequency. For statistical analysis, the Statistics Package for Social Sciences, version 12.0 for Windows (SPSS Inc., Chicag, IL, USA) was used. One-way analysis of variance (ANOVA) or Mann-Whitney U test was performed to analyze the significant differences of continuous data between EJ- and TE-group. Chi-square test was performed to analyze the significant differences of categorical data. A *P*-value <0.05 was considered significantly different between two groups.

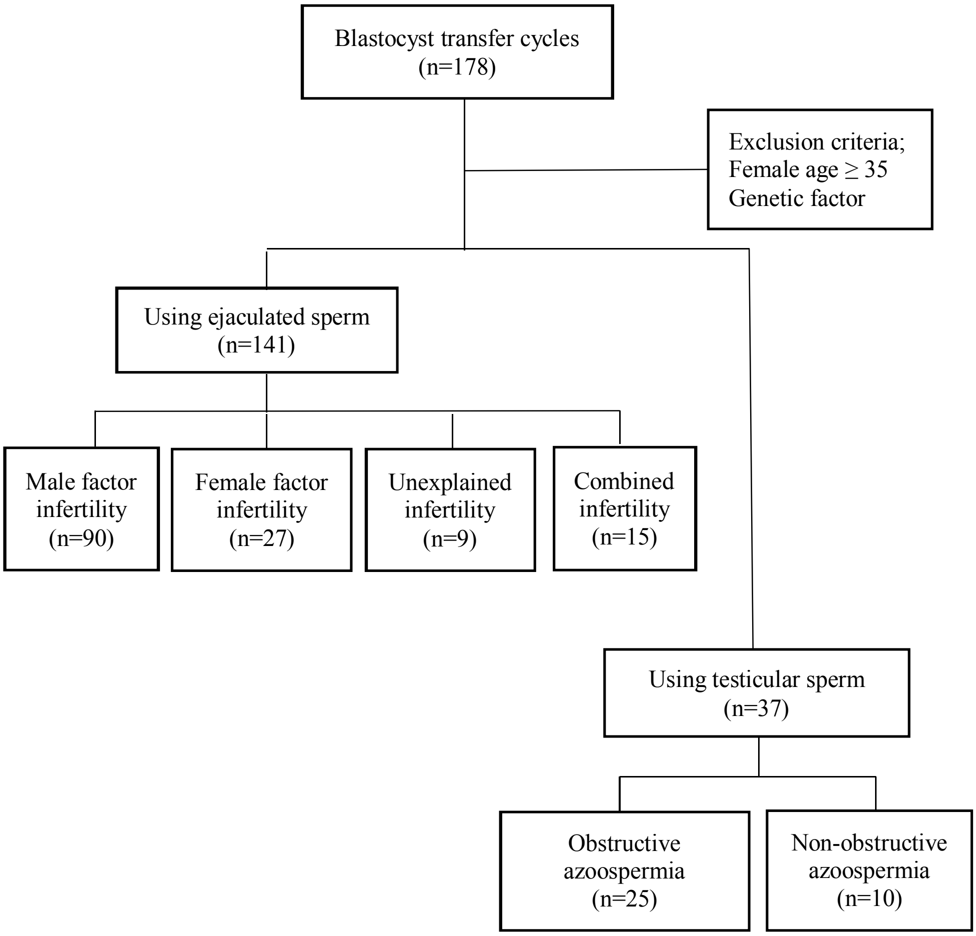

Figure 3-1. A schematic diagram for patient selection to evaluate the effect of testicular sperm on blastocyst formation and pregnancy rate.

Blastocyst grading

Size of blastocyst

	Size
Early blastocyst	1
Blastocyst	2, 3
Expended blastocyst	4
Hatching blastocyst	5
Hatched blastocyst	6

Quality of inner cell mass (ICM) and trophectoderm (TE)

ICM	TE
A	A
B	B
C	C

Blastocyst grade = Size, ICM, TE

Figure 3-2. Blastocyst grading criteria. Blastocysts were classified into six grades based on the degree of expansion and the status of hatching.

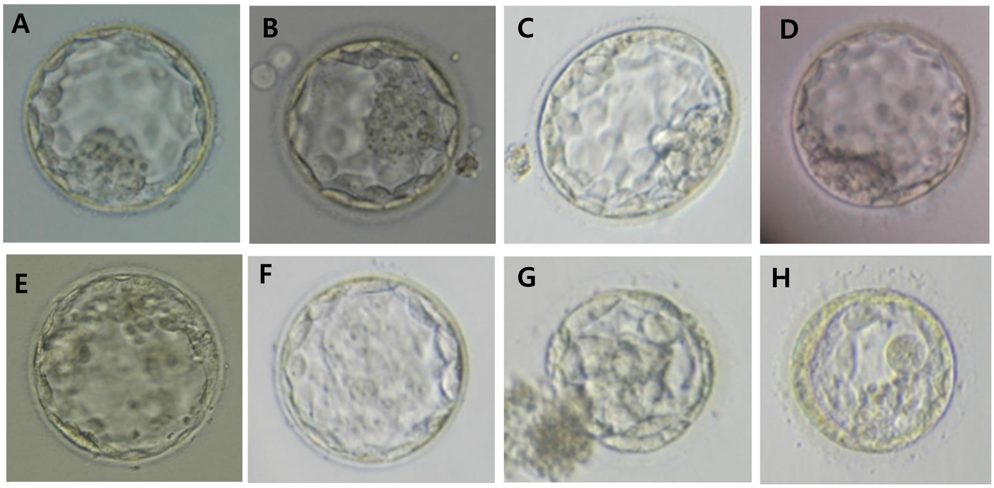

Figure 3-3. Photomicrographs of blastocysts with different quality. A. 4AA blastocyst; B. 5AA blastocyst; C, D. 4BA blastocyst; E. 4BC blastocyst; F. 5CB blastocyst has no ICM; G. 1BB blastocyst; H. 2BC blastocyst.

3-3. RESULTS

1. Fertilization after ICSI using ejaculated or testicular sperm

Characteristics of patients and variables of cycles were compared between both groups (Table 3-1). Body mass indexes (BMI) of female partners were significantly higher in TE-group than in EJ-group (22.0 ± 2.9 vs. 20.9 ± 2.9 kg/m2, $p<0.05$). Baseline characteristics of patients except for BMI, such as age of female (31.2 ± 2.5 vs. 30.2 ± 2.9 years old) and male partners (34.7 ± 3.7 vs. 35.5 ± 5.4 years old), duration of infertility (3.6 ± 2.3 vs. 2.9 ± 2.3 years), were not different between the both groups. And cycle variables, such as FSH level on day 2 or 3 of the menstrual cycles (7.4 ± 2.5 vs. 7.0 ± 1.7 mIU/ml), duration of controlled ovarian stimulation (COH) (9.9 ± 1.7 vs. 10.2 ± 1.7 days), total dosage of FSH used for COH (2,317.6 ± 942.9 vs. 2,457.8 ± 909.5 IU), E2 level on day of hCG administration (3,097.1 ± 1,995.8 vs. 3,297.5 ± 1,669.7 pg/ml), were comparable between the both groups.

Normal fertilization (2PN and the second polar body) rate was compared between the EJ- and TE-groups (Table 3-2). Ejaculated sperm were injected into oocytes in 141 cycles and testicular sperm in 37 cycles. A total of 2,672 oocytes (19.0 ± 8.1 per cycle) were retrieved and ejaculated sperm were injected into 2,116 oocytes (15.0 ± 6.8 per cycle) in the EJ-group. Among the injected oocytes, normal fertilization was observed in 1,617 oocytes (11.5 ± 5.3 per cycle). Seven hundred and ninety-seven oocytes (21.5 ± 6.1 per cycle) were retrieved and testicular sperm were injected into

620 oocytes (16.8 ± 4.7 per cycle) in the TE-group. Normal fertilization was observed in 437 oocytes (11.8 ± 4.8 per cycle). Significantly more oocytes were retrieved in the TE-group than in the EJ-group ($P < 0.05$). There was no significant difference in the rate of metaphase II oocytes (80.0 ± 12.7% vs. 79.5 ± 14.3%, respectively). Normal fertilization rate was significantly higher ($P < 0.05$) in EJ-group (77.3 ± 15.0%) than in TE-group (69.6 ± 17.0%). Embryos were transferred on day five in all cycles. An average of 2.5 ± 0.8 embryos was transferred in the EJ-group and 2.3 ± 0.8 embryos in the TE-group. The number of transferred embryos was not different between the two groups. Implantation was confirmed by examining the number of gestational sacs. Implantation rate was 22.8% in the EJ-group and 24.7% in the TE-group. Implantation rate was not different between two groups.

In the TE-group, fertilization was compared between patients with obstructive azoospermia and patients with non-obstructive azoospermia (Table 3-6). Clinical outcomes of patients with retrograde ejaculation were excluded in comparison between obstructive and non-obstructive azoospermia patients. Female age was not different between patients with obstructive azoospermia and patients with non-obstructive azoospermia (30.7 ± 2.7 vs. 29.2 ± 3.4). In total, 544 oocytes (21.8 ± 6.3 per cycle) were retrieved and testicular sperm were injected into 427 oocytes (17.1 ± 5.3 per cycle) in patients with obstructive azoospermia. Three hundred and eighteen oocytes were normally fertilized (12.7 ± 5.3 per cycle). In patients with non-obstructive azoospermia, 219 oocytes (21.9 ± 6.0 per cycle) were retrieved and testicular sperm were injected into 160 oocytes (10 ± 3.1 per cycle). Normal fertilization was observed in 101 oocytes (10.1 ± 2.7 per cycle). The rate of normal fertilization was not different between patients with obstructive azoospermia and patients with non-obstructive azoospermia (73.3 ± 16.3% vs 63.9 ± 15.7%, respectively).

2. Embryo quality and development to blastocyst stage after ICSI using ejaculated sperm or testicular sperm

Among the fertilized oocytes, some zygotes were frozen at the pronuclear stage, and the remaining zygotes were cultured for five days. Embryo quality was observed on day three and just before embryo transfer on day five (Table 3-3). In total, 1,171 zygotes were cultured for five days in the EJ-group. Of these, 298 embryos (2.1 ± 1.8 per cycle) were good quality embryos on day three. In the TE group, 326 zygotes were cultured for five days. Of these, 66 embryos (1.8 ± 1.9 per cycle) were assessed as good quality. There was no difference in embryo quality on day three between the EJ- and TE-groups. In the EJ-group, 58 embryos (0.4 ± 0.7 per cycle) were good quality embryos on day five after oocyte retrieval. Eighteen embryos (0.5 ± 0.7 per cycle) showed good quality in the TE-group on day five. Similar to embryo quality on day three, there were no differences in embryos qualities on day five between both groups. In the TE-group, embryo quality was not different between patients with obstructive azoospermia and those with non-obstructive azoospermia patients on days three and five (Table 3-7).

Blastocyst formation rate was compared between the EJ- and TE-group (Table 3-4; Fig. 3-4). In the EJ-group, embryos did not develop to blastocysts in seven of 141 cycles (5.0%). In total, 1,171 embryos were cultured for five days and 532 embryos (3.8 ± 2.1 per cycle) developed to blastocyst stage (46.1 ± 24.7%). Among the blastocysts, 188 embryos (1.3 ± 1.5 per cycle) were expanded or hatching blastocysts. In the TE-group, embryos did not develop to blastocysts in two of 37 cycles (5.4%). A total of 326 embryos were cultured for five days and 154 embryos (4.2 ± 2.2 per cycle) developed to blastocyst stage. Sixty-one embryos (1.6 ± 1.5 per cycle) were expanded or hatching blastocysts. The blastocyst formation rate was not different between the EJ- and TE-groups (46.1 ± 24.7% vs. 47.5 ± 21.6%, respectively). In the TE-group, the blastocyst formation rate and the rate of embryos that were developed to expanded or hatching blastocysts were not different between patients with obstructive azoospermia and patients with non-obstructive azoospermia (Table 3-8).

3. Pregnancy outcomes after ICSI using ejaculated sperm or testicular sperm

Clinical pregnancy was confirmed in 63 cycles of the EJ-group (44.7%) and in 16 cycles of the TE-group (43.2%) (Table 3-5). There was no significant difference in clinical pregnancy rates between the both groups. Pregnancy was not established in cycles in which embryos did not develop to blastocyst stage (7 cycles in EJ-group and 2 cycles in TE-group). In the EJ-group, 47 babies were delivered in 46 cycles (76.7%). One pregnancy was twin pregnancy (1.7%) and 45 were singleton pregnancies (75.0%). Thirteen pregnancies resulted in miscarriages within the first trimester of pregnancy (21.7%). One pregnancy was ectopic and follow-ups of three pregnancies were unsuccessful because contact with the patients was lost. In the TE-group, 11 babies were delivered in ten cycles (66.7%). One pregnancy was twin pregnancy (6.7%) and nine were singleton pregnancies (60.0%). Five pregnancies ended in miscarriage (33.3%), and follow-up was failed in one pregnancy. Live-birth rate and miscarriage rate were not different between the EJ- and TE-groups.

Pregnancy outcomes were compared between patients with obstructive azoospermia and patients with non-obstructive azoospermia (Table 3-9). Clinical pregnancies were confirmed in 11 cycles and implantation rate was 24.1% in patients with obstructive azoospermia. Five babies were delivered in five cycles, five pregnancies resulted in miscarriages and follow-up of one pregnancy was failed. In patients with non-obstructive azoospermia, five clinical pregnancies were established. The implantation rate was 32.0% and six babies were delivered in those cycles. Live-birth rate and miscarriage rate were not different between patients with obstructive azoospermia and patients with non-obstructive azoospermia.

Table 3-1. Comparison of patients' characteristics between two groups

	ICSI using ejaculated sperm	ICSI using testicular sperm	*p*-value
No. of cycles	141	37	
Age of female partner (year) *	31.2±2.5	30.2±2.9	0.057
BMI of female partner (kg/m^2) *	20.9±2.7	22.0±2.9	0.025
Age of male partner (year) *	34.7±3.7	35.5±5.4	0.794
Duration of infertility (year) *	3.6±2.3	2.9±2.3	0.070
FSH level on day 2 or 3 of the menstrual cycle (mIU/ml) *	7.4±2.5	7.0±1.7	0.428
Duration of controlled ovarian hyperstimulation (days) *	9.9±1.7	10.2±1.7	0.344
Dosage of FSH used to stimulation (IU) *	2,317.6±942.9	2,457.8±909.5	0.353
E2 level on hCG administration day (pg/ml) *	3,097.1±1,995.8	3,578.9±1,759.1	0.075
No. of retrieved oocytes	2,672	797	0.014
No. of injected oocytes	2,116 (79.2)	620 (77.8)	0.026

* Values represent means ± SD

Table 3-2. Fertilization after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

	ICSI using ejaculated sperm	ICSI using testicular sperm	*p*-value
No. of cycles	141	37	
No. of retrieved oocytes [*]	2,672 (19.0±8.1)	797 (21.5±6.1)	0.014
No. of injected oocytes [*]	2,116 (15.0±6.8)	620 (16.8±4.1)	0.026
No. of normally fertilized oocytes (2PN) [*]	1,617 (11.5±5.3)	437 (11.8±4.8)	0.688
Rate of normally fertilized oocytes (%) [**]	77.3±15.0	69.6±17.0	0.017
No. of cultured embryos [*]	1,171 (8.3±2.3)	326 (8.8±2.0)	0.103
No. of transferred embryos [*]	346 (2.5±0.8)	85 (2.3±0.8)	0.297
No. of gestational sacs (%[***])	79 (22.8)	21 (24.7)	0.716

[*] Values in parentheses represent mean number of oocytes or embryos per cycle. Values are expressed as mean ± SD.

[**] Values are the average of the fertilization rate after the fertilization rate is calculated for each cycle. Values are expressed as mean ± SD.

[***] Values are the percentage of gestational sacs to transferred embryos.

Table 3-3. Embryo quality on day 3 and day 5 after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

		ICSI using ejaculated sperm	ICSI using testicular sperm	*p*-value
Day 3	Good quality embryos [a] (%)	2.1±1.8 (27.7±24.0)	1.8±1.9 (19.5±19.9)	0.184
	Fair quality embryos [b] (%)	3.9±2.2 (45.9±23.1)	4.7±1.9 (53.6±21.6)	0.025
	Poor quality embryos [c] (%)	2.3±2.1 (26.7±20.3)	2.4±1.9 (26.8±21.1)	0.678
Day 5	Good quality embryos [d] (%)	0.4±0.7 (5.7±9.8)	0.5±0.7 (5.5±8.8)	0.523
	Fair quality embryos [e] (%)	1.2±1.1 (15.2±14.5)	1.5±1.3 (17.4±14.2)	0.142
	Poor quality embryos [f] (%)	6.7±2.7 (79.3±19.5)	6.8±2.0 (77.1±15.3)	0.654

Values are mean number of embryos per cycle and expressed as means± SD.

Values in parentheses are the average of the rates after the rates of good, fair or poor quality embryos are calculated for each cycle. Values are expressed means ± SD.

[a] embryos with seven or more even-sized blastomeres and less than 10% cytoplasmic fragmentation on three day after oocyte retrieval

[b] embryos with six or more uneven-sized blastomeres and 10 to 40% cytoplasmic fragmentation on three day after oocyte retrieval

[c] embryos with five or less blastomeres or more than 40% cytoplasmic fragmentation on three day after oocyte retrieval

[d] expanded, hatching or hatched blastocysts with ICM consisted of tightly packed cells on five day after oocyte retrieval

[e] blastocysts with ICM consisted of several loose cells and trophectoderm consisted of several cells on five day after oocyte retrieval

[f] embryos that did not develop to blastocysts, early, middle blastocysts or blastocysts with ICM consisted of very few cells and trophectoderm with very few large cells on day five after oocyte retrieval

Table 3-4. Development to blastocyst stage after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

	ICSI using ejaculated sperm	ICSI using testicular sperm	*p*-value
No. cultured embryos [*]	1,171 (8.3±2.3)	326 (8.8±2.0)	0.103
No. of blastocysts [*]	532 (3.8±2.1)	154 (4.2±2.2)	0.348
Rate of blastocysts (%) [**]	46.1±24.7	47.5±21.6	0.118
No. of expanded or hatching blastocysts [*]	188 (1.3±1.5)	61 (1.6±1.5)	0.994
Rate of expanded or hatching blastocysts (%) [*]	16.8±19.0	18.4±16.1	0.407

[*] Values in parentheses represent mean number of embryos or blastocysts per cycle. Values are expressed as mean ± SD.

[**] Values are the average of the blastocyst formation rate after the blastocyst formation rate is calculated for each cycle. Values are expressed as mean ± SD.

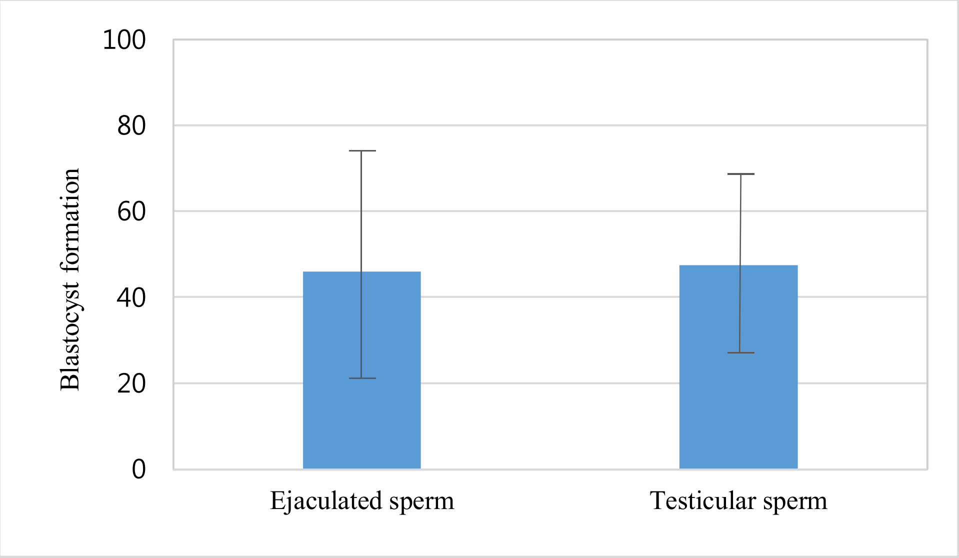

Figure 3-4. Comparison of blastocyst formation rate between ejaculated sperm and testicular sperm group. There was no significant difference.

Table 3-5. Pregnancy outcomes after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

	ICSI using ejaculated sperm	ICSI using testicular sperm	*p*-value
Transfer cycles	141	37	
Transfer cycles with blastocysts	134 (95.0)	35 (94.6)	1.000
Clinical pregnancies (%)	63 (44.7)	16 (43.2)	0.876
Follow-up loss	3	1	
Miscarriages (%[*])	13 (21.7)	5 (33.3)	0.335
Ectopic pregnancies	1	0	
Deliveries (%[*])	46 (76.7)	10 (66.7)	0.537
Singleton pregnancy (%[*])	45 (75.0)	9 (60.0)	
Twin pregnancy (%[*])	1 (1.7)	1 (6.7)	
Delivered babies	47	11	

[*] Values are the percentage of miscarriages or deliveries to clinical pregnancy excluding follow-up loss.

Table 3-6. Fertilization after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

	ICSI using ejaculated sperm		ICSI using testicular sperm		*p*-value
	Female factor	Male factor	Obstructive azoospermia	Non-obstructive azoospermia	
No. of cycles	27	90	25	10	
Average female age [*]	31.3±2.4	31.0±2.5	30.7±2.7	29.2±3.4	0.285
No. of retrieved oocytes [*]	514 (19.0±9.5)	1,678 (18.6±7.4)	544 (21.8±6.3)	219 (21.9±6.0)	0.219
No. of injected oocytes [*]	422 (15.6±8.4)	1,299 (14.4±5.8)	427 (17.1±5.3)	160 (16.0±3.1)	0.262
No. of normally fertilized oocytes (2PN) [**]	352 (12.0±6.1)	986 (11.0±4.7)	318 (12.7±5.3)	101 (10.1±2.7)	0.437
Rate of normally fertilized oocytes (%) [*]	78.1±15.1	76.9±16.0	73.3±16.3	63.9±15.7	0.229
No. of cultured embryos [*]	220 (8.1±1.4)	747 (8.3±2.5)	223 (8.9±2.1)	90 (9.0±1.4)	0.256
No. of transferred embryos [*]	69 (2.6±0.7)	215 (2.4±0.7)	54 (2.2±0.7)	25 (2.5±1.0)	0.308
Gestational sacs (%[***])	12 (17.4)	53 (24.6)	13 (24.1)	8 (32.0)	0.460

[*] Values in parentheses represent mean number of oocytes or embryos per cycle. Values are expressed as mean ± SD.

[**] Values are the average of the fertilization rate after the fertilization rate is calculated for each cycle. Values are expressed as mean ± SD.

[***] Values are the percentage of gestational sacs to transferred embryos.

Table 3-7. Embryo quality on day 3 and day 5 after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

		ICSI using ejaculated sperm		ICSI using testicular sperm		*p*-value
		Female factor	Male factor	Obstructive azoospermia	Non-obstructive azoospermia	
Day 3	Good quality embryos [e] (%)	1.7±1.7 (20.5±19.3)	2.4±1.9 (32.1±25.9 [a])	1.6±1.9 (17.5±21.0 [b])	2.6±2.0 (27.4±16.9)	0.067
	Fair quality embryos [f] (%)	3.9±1.8 (48.6±22.2)	3.6±2.4 (42.4±23.4)	4.7±2.3 (52.3±22.1)	4.6±0.8 (52.8±15.2)	0.082
	Poor quality embryos [g] (%)	2.5±1.5 (30.9±17.3)	2.3±2.3 (25.9±21.3)	2.6±2.0 (30.3±22.6)	2.6±2.0 (30.3±22.6)	0.319
Day 5	Good quality embryos [h] (%)	0.5±0.9 (5.6±10.9)	0.4±0.7 (6.0±10.2)	0.5±0.8 (5.9±10.1)	0.5±0.5 (5.5±5.8)	0.808
	Fair quality embryos [i] (%)	1.8±1.2 [a] (22.6±15.4 [c])	1.1±1.1 [b] (13.8±14.1 [d])	1.5±1.2 (16.8±12.7)	1.8±1.4 (21.1±18.1)	0.016
	Poor quality embryos [j] (%)	5.9±2.0 (71.7±19.3)	6.8±2.9 (80.3±19.8)	6.9±1.9 (77.3±13.0)	6.7±2.4 (73.4±20.1)	0.362

Values are mean number of embryos per cycle and expressed as means± SD

Values in parentheses are the average of the rates after the rates of good, fair or poor quality embryos are calculated for each cycle. Values are expressed means ± SD.

[a] vs. [b] $p = 0.006$, [c] vs. [d] $p = 0.007$

[e] embryos with seven or more even-sized blastomeres and less than 10% cytoplasmic fragmentation on three day after oocyte retrieval

[f] embryos with six or more uneven-sized blastomeres and 10 to 40% cytoplasmic fragmentation on three day after oocyte retrieval

[g] embryos with five or less blastomeres or more than 40% cytoplasmic fragmentation on three day after oocyte retrieval

[h] expanded, hatching or hatched blastocysts with ICM consisted of tightly packed cells on five day after oocyte retrieval

[i] blastocysts with ICM consisted of several loose cells and trophectoderm consisted of several cells on five day after oocyte retrieval

[j] early, middle blastocysts or blastocysts with ICM consisted of very few cells and trophectoderm with very few large cells on day five after oocyte retrieval

Table 3-8. Development to blastocyst stage after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

	ICSI using ejaculated sperm		ICSI using testicular sperm		p-value
	Female factor	Male factor	Obstructive azoospermia	Non-obstructive azoospermia	
No. cultured embryos *	115 (8.1±1.4)	747 (8.3±2.5)	223 (8.9±2.1)	90 (9.0±1.4)	0.256
No. of blastocysts *	115 (4.3±1.9)	347 (3.9±2.2)	103 (4.1±2.1)	44 (4.4±2.8)	0.769
Rate of blastocysts (%) **	51.8±19.4	47.2±24.9	46.1±19.1	50.3±28.9	0.700
No. of expanded or hatching blastocysts *	44 (1.6±1.8)	116 (1.3±1.4)	42 (1.7±1.6)	18 (1.8±1.5)	0.502
Rate of expanded or hatching blastocysts (%) *	20.4±21.9	16.5±18.5	18.5±16.0	20.8±17.5	0.709

* Values in parentheses represent mean number of embryos or blastocysts per cycle. Values are expressed as mean ± standard deviation.

** Values are the average of the blastocyst formation rate after the blastocyst formation rate is calculated for each cycle. Values are expressed as mean ± SD.

Table 3-9. Pregnancy outcomes after intracytoplasmic sperm injection (ICSI) using ejaculated or testicular sperm

	ICSI using ejaculated sperm		ICSI using testicular sperm		p-value
	Female factor	Male factor	Obstructive azoospermia	Non-obstructive azoospermia	
Transfer cycles	27	90	25	10	
Transfer cycles with blastocysts	27 (100.0)	87 (96.7)	25 (100.0)	8 (80.0)	
Clinical pregnancies (%)	9 (33.3)	43 (47.8)	11 (44.0)	5 (50.0)	0.599
Follow-up loss	1	1	1		
Miscarriages (%[*])	2 (25.0)	9 (21.3)	5 (50.0)		0.149
Ectopic pregnancies		1			
Deliveries (%[*])	6 (75.0)	32 (76.2)	5 (50.0)	5 (100.0)	0.184
Delivered babies	7	32	5	6	

[*] Values are the percentage of miscarriages or deliveries to clinical pregnancy excluding follow-up loss.

3-4. Discussion

For pregnancy in infertile couples due to azoospermia, ICSI is widely performed using TE. However, in these couples, blastocyst transfer is not widely performed compared to couples for whom ICSI is performed using EJ. This may be due to the fact that poor quality of TE may affect embryonic development to blastocyst stage (Miller et al., 2001). Recently, it has been reported that clinical outcomes of ICSI using TE are comparable to those of ICSI using EJ (Braga et al., 2013; Naru et al., 2008; Nilsson et al., 2007; Xie et al., 2014). Furthermore, these studies also suggest that the ability of TE to support embryonic development to blastocyst stage is comparable to that of EJ, and clinical outcomes of blastocyst transfer in cycles in which ICSI is performed using TE are not different to those in cycles where ICSI is performed using EJ. Those are compatible with the results of this study. Both TE and EJ supported the development of fertilized oocyte to blastocyst with similar capacity.

The relationship between the clinical outcomes of ICSI and the sperm sources has long been controversial and unclear. Recent studies have shown that clinical outcomes of ICSI were better when ICSI was performed using EJ than when ICSI was performed using TE (Balaban et al., 2001; Rossi-Ferragut et al., 2003; Ghazzawi et al., 1998; Pasqualotto et al., 2002). According to these studies, fertilization, blastocyst formation, and implantation rates were higher, qualities of embryos were better, and miscarriage rate was lower when ICSI was performed using EJ than when it was performed using TE. More recently, however, conflicting studies have been reported. Nilsson et al reported that clinical outcomes of single blastocyst transfer after ICSI using epididymal or TE were similar to those of standard IVF or ICSI using EJ (Nilsson et al. 2007). However, the authors did not distinguish between the clinical outcomes of epididymal sperm and those of TE. Therefore, if the clinical outcomes of TE were distinct to those of epididymal sperm, it might also be different from those of EJ. Naru et al. observed no difference in pregnancy and miscarriage rates between ICSI cycles using EJ and those using TE (Nilsson et al., 2007). Furthermore, Braga et al. reported significantly higher implantation rate in cycles using TE than in cycles using EJ or epididymal sperm (Braga et al., 2013). Xie et al. also reported higher implantation rates in cycles using TE than in cycles using EJ (Xie et al., 2014). Although the reasons are unclear, there have been conflicting studies on the relationship between clinical outcomes of ICSI and sperm sources. These conflicting results may be caused by the different procedures of TE retrieval or different characteristics of patients who underwent TE retrieval. Testicular sperm can be retrieved through a variety of medical procedures. However, two procedures are mainly performed: needle biopsy (testicular sperm aspiration [TESA]) or open testicular biopsy (testicular sperm extraction [TESE]). Retrieval of TE, and the quantity and quality of retrieved TE may be affected by retrieval procedures. Several studies have reported that the clinical outcome of ICSI using EJ was better than those of using TE. In those studies, testicular sperm was recovered by TESA rather than TESE. However, in studies that reported the outcomes of ICSI using TE comparable to those using EJ, the frequency of studies using TESE were similar to that using TESA. Hauser et al. (2006) conducted TESE and TESA simultaneously in men with non-obstructive azoospermia and reported that TESE was more advantageous than TESA in retrieving TE. Although the results of the study were limited to men with non-obstructive azoospermia, the retrieval rate of TE, the retrieval rate of motile sperm and the quantity of retrieved sperm were higher in TESE than in TESA. Therefore, medical procedures involving testicular sperm retrieval may affect the clinical outcome of ICSI. Testicular histopathology of men with azoospermia was not described in most of the studies comparing clinical outcomes of ICSI dependent on sperm sources. The sperm quantity may be different in patients with obstructive azoospermia and those with non-obstructive azoospermia. The clinical outcomes of ICSI can also be affected by the sperm quality. Fertilization rates after ICSI were higher when motile sperm were injected into oocytes than when immotile sperm were injected (Nagy et al., 1998; Shulman et al., 1999). The more sperm available to ICSI, the more appropriate sperm for ICSI will be selected. Balaban et al. (2001) compared the clinical outcomes of ICSI between men with obstructive azoospermia and men with non-obstructive azoospermia. According to their study, the clinical outcomes of ICSI using TE retrieved from men with non-obstructive azoospermia were decreased compared to those using TE retrieved from men with obstructive azoospermia.

When ICSI was performed using TE retrieved from men with obstructive azoospermia, the clinical outcomes of ICSI were comparable to those of ICSI using either ejaculated or epididymal sperm. Taken together, these results indicate that the histopathology of men with azoospermia may have significant impacts on the clinical outcomes of ICSI using TE. Therefore, clinical outcomes of men with obstructive azoospermia should be analyzed separately from those of men with non-obstructive azoospermia. In the present study, clinical outcomes of men with obstructive azoospermia and those of men with non-obstructive azoospermia were compared but no differences were observed between them. This could be attributed to the low number of men with non-obstructive azoospermia in this study.

Not all clinical outcomes of ICSI using TE were comparable to those of ICSI using EJ. Recent studies have reported decreased fertilization rates in ICSI cycles using TE (Goker at al., 2002; Palermo et al., 1999; Pasqualotto et al., 2002; Xie et al., 2014). In this study, fertilization rates were significantly lower in ICSI cycles using TE than in cycles using EJ. However, fertilization rates were decreased only in cycles involving men with non-obstructive azoospermia when clinical outcomes were analyzed separately in men with obstructive and non-obstructive azoospermia. In ICSI cycles of men with obstructive azoospermia, fertilization rates were comparable to those of ICSI cycles using EJ. Consistent results were also reported in other studies (Balaban et al., 2001; Esteves et al., 2014; Tehraninejad et al., 2012). Fertilization rate was significantly lower in ICSI cycles of men with non-obstructive azoospermia than in cycles of men with obstructive azoospermia. These decreased fertilization rate in men with non-obstructive azoospermia may be consequent on centrosome defects of TE retrieved from men with non-obstructive azoospermia (Asch et al., 1995; Simerly et al. 1997).

In this study, clinical outcomes of blastocyst transfer performed for quite a long time, blastocyst transfer cycles of 11 years were analyzed. During this period, blastocyst transfer was performed very carefully, especially in ICSI cycles using TE, because the age of female partners was increased (form 34.7 ± 4.4 to 37.3 ± 4.6 years old) and the number of retrieved oocytes was decreased (from 11.6 ± 9.5 to 9.0 ± 8.4 per cycle). Therefore, such a long time was required until the enough data of blastocyst transfer were collected in ICSI cycles using TE. Although many techniques were changed in IVF and ICSI during this period, ICSI was performed in the same manner in all ICSI cycles using EJ or TE. The medical equipment used to ICSI, such as micromanipulators and microscopes, were also developed and improved during the period but we performed ICSI using same equipment. And three embryologists performed ICSI mainly during this period. Their experience of performing ICSI is more than ten years. They have also performed various assisted reproductive techniques but, mainly ICSI. The preparation method of EJ or TE for ICSI was not changed. EJ was prepared for ICSI by swim-up methods and TE by squeezing seminiferous tubules using two pairs of fine forceps under dissecting microscope. Surgical method for collecting testicular tissue has not changed.

Testicular tissue was collected from men with obstructive azoospermia by conventional testicular biopsy. Microdissection TESE using microscope (micro-TESE) was performed for collecting testicular tissue in men with non-obstructive azoospermia. We have maintained many factors that can affect the clinical outcomes of ICSI, such as the manner of ICSI, equipment, embryologists, sperm preparation methods and surgical method for collecting testicular tissue, largely unchanged and constant during this period. Therefore, in my opinion, the long data collection period will have little impact on the results of this study.

In this study, the embryo quality was analyzed and compared between two groups, which was not performed in other studies. The rate of good quality embryos was low on day 3 or day 5. The low rate of good quality embryos seems to be due to our strict evaluation of embryo quality. The rates of good or poor quality embryos were similar between two groups on 3 or 5 days after oocyte retrieval. The rate of fair quality embryos was higher in TE-group than in EJ-group on day 3. However, the rate was not different between two groups on day 5. The correlation between cleavage-stage embryo quality and blastocyst quality was failed to analyzed and compared, since the number of embryos was too small to analyze and compare it. In order to perform blastocyst transfer, the number of cultured embryos and the blastocyst formation rate seem to be more important rather than embryo quality on day 3 or the correlation of between cleavage-stage embryo quality and blastocyst quality. This study showed that TE had the potential similar to that of EJ in supporting embryonic development to blastocysts. These results are consistent with several studies reported recently (Nilsson et al., 2007; Naru et al., 2008; Braga et al., 2013; Xie et al., 2014). Blastocyst transfer should be actively considered in cycles using TE as well as in cycles using EJ. It is already well known that pregnancy rate can be improved by performing blastocyst transfer (Baker et al., 2007; Papanikolaou et al., 2008). Recently, blastocyst transfer has been extensively performed and its implementation is increasing. However, until now, it appears that this is not the case in the cycles using TE. The improvement of pregnancy rates were also reported in cycles using TE when blastocysts were transferred (Virant-Klun et al., 2003). In cycles using TE, blastocyst transfer should be performed more extensively than now. Of course, the appropriate number of oocytes should be retrieved although it is not easy since the number of the retrieved oocyte is decreasing due to the rise in female age and other causes.

In summary, the present study showed that the potential of TE supporting embryonic development to blastocyst stage was comparable to that of EJ. The clinical outcomes of blastocyst transfer in ICSI cycles using TE were similar to those in ICSI cycles using EJ. Therefore, the present study suggests that blastocyst transfer may be considered when the appropriate numbers of oocyte are retrieved and motile sperm are identified in ICSI cycles using TE.

Chapter 4

Effect of Oxygen Concentration on Embryo Quality and Pregnancy rate

4-1. INTRODUCTION

Since the first baby was born by the assisted reproductive technologies (ART) in 1978, more than 6 million babies have been born worldwide. One of the key factors for the success of ART, defined as delivery of a healthy child, is the quality of embryo culture environment. Specifically, the conditions within laboratory incubator, such as oxygen tension, CO_2 concentration, and temperature stability, can all impact embryo development (Gardner et al., 2012; Higdon et al., 2008; Karagenc et al., 2004; Scott et al., 1993; Swain, 2015). Usually in an IVF laboratory, gametes and embryos are cultured in sodium bicarbonate buffered media which maintains optimal pH under the 5-6% carbon dioxide and atmospheric oxygen concentrations. Previous studies have reported that the oxygen concentration in the oviducts and uterus of most mammalian species is 2%–8% (Fischer and Bavister, 1993; Mastroiannil and Jones, 1965; Ottosen et al., 2006). So, when gametes are exposed to atmospheric oxygen concentrations (20% oxygen concentration), gametes and embryos could be under the oxidative stress caused by free oxygen radicals (Fischer and Bavister, 1993). The free oxygen radicals, such as superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, organic peroxide radicals and singlet molecular oxygen, may cause damage to the cell membrane and DNA fragmentation in somatic cells, and participate in the process of apoptosis. In the several studies, the adverse effects of free radicals on the human and mouse embryos have been reported (Agarwal et al., 2006; Cebral et al., 2007). Oxygen tensions can change the gene expression pattern of embryos (Giritharan et al., 2007). Rinaudo et al. (2006) speculated that the embryos culturing in 20% oxygen results in greater perturbations in the global pattern of gene expression than when the embryos are cultured in 5% oxygen concentration.

In vitro development of porcine oocytes to the blastocyst stage was significantly accelerated under 5% oxygen, a concentration of oxygen similar to that in the oviduct and uterus. Moreover, the average number of cells in the blastocyst was slightly higher in the culture under 5% oxygen concentrations versus 20% oxygen concentrations (Dumoulin et al., 1999). Hydrogen peroxide (H_2O_2) concentration of porcine embryos cultured in 20% oxygen was higher than that of embryos cultured in 5% oxygen (Kitagawa et al., 2004). Yang et al. (1998) reported that reactive oxygen species (ROS) such as hydrogen peroxide increased fragmentation of cultured embryos, and then they suggested that increased ROS in atmospheric culture condition can induces embryonic cytoplasmic fragmentation and apoptosis. Numerous studies have demonstrated that 20% oxygen concentration, atmospheric oxygen, is damaging to embryo development. On the other hand, other studies have reported that elevated oxygen concentration does not alter embryo development on day 2 or 3 in human IVF-ET cycles. Karagenc et al. (2004) has concluded that 20% oxygen concentration does not effect on mouse embryo development not only cleavage stage but also blastocyst in vitro culture (Bahçeci et al., 2005; Karagenc et al., 2004). However, the question remains, does the 20% oxygen concentration adversely effect on development of preimplantation embryos. Besides, many factors such as cell density media volume, oil covering etc, are determined the real level in media and not defined finely.

In order to achieve successful pregnancy rate, oocytes and embryos must culture in the optimal condition, more physiological condition, during whole IVF-ET procedure. In this study, the effect of oxygen concentration on embryo quality and pregnancy rate in IVF-ET cycles was examined.

4-2. MATERIALS AND METHODS

1. Patients, Controlled ovarian hyperstimulation, and Oocyte Retrieval

In total 375 IVF-ET cycles, retrieved oocytes and embryos were cultured in two different gas phases condition – the physiological condition group with 5% O_2, 5% CO_2, and 90% N_2 (n=170) and the atmospheric condition group with 5% CO_2 and air balance (n=205) (fig. 4-1). When the female age was more than 40 years old, the cycles was not included in this study. Donor cycles and preimplantation genetic diagnosis (PGD) cycles were also excluded.

Ovarian stimulation was carried out using gonadotropin releasing hormone (GnRH) agonist/antagonist, human menopausal gonadotropin (hMG), and human recombinant follicle stimulating hormone (FSH). Human chorionic gonadotropin (hCG) was administered when optimal follicle development was achieved, as evaluated by serial transvaginal ultrasound and estrogen determinations. Oocyte retrieval was performed via a transvaginal approach with sonographic guidance 34 hours after hCG injection.

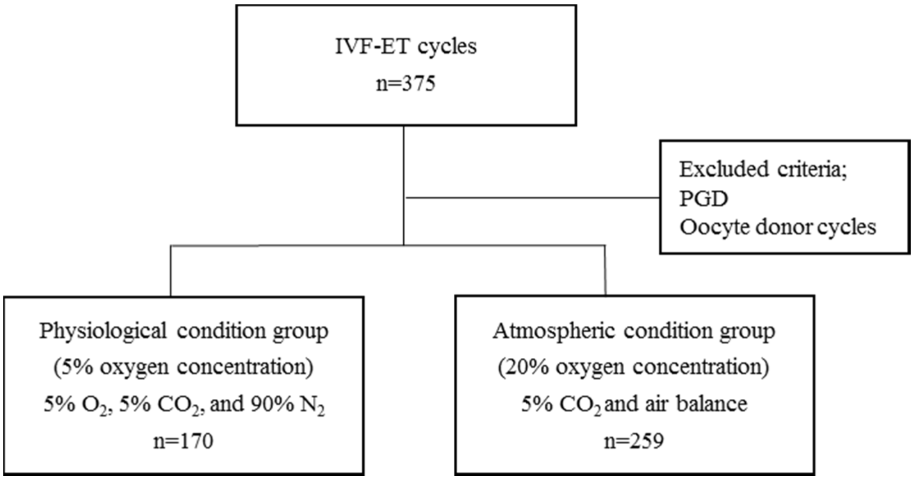

Figure 4-1. A schematic diagram for patient selection to evaluate the effect of oxygen concentration.

2. Sperm preparation, in vitro fertilization and embryo culture

Semen samples were collected immediately after oocyte pick-up. Sperm concentration and motility were analyzed by a light microscopy (BI41T, OLYMPUS, Japan). Sperm was prepared either by swim-up method or by the density gradient centrifugation method. In conventional IVF, the oocytes allocated to IVF were maintained in fertilization medium until the sperm samples were prepared. The oocyte–cumulus complexes were inseminated with 50,000 or more motile spermatozoa per insemination dish. After 16-18 h, the inseminated oocytes were stripped from the cumulus cells to check for fertilization and the maturity status of the non-fertilized. For ICSI cycles, cumulus cells were removed approximately 1 hour after oocyte retrieval using 80IU/mL of hyaluronidase. The remaining cells were mechanically removed by gentle pipetting with glass capillaries. Among the oocytes allocated to ICSI, mature metaphase II oocytes were selected and injected with sperm. ICSI was performed at least 1 hour after removing the cumulus cells. Fertilization was assessed 16 hours after ICSI. Only oocytes with two pronuclei and the second polar body were assessed as normally fertilized oocytes. For the effect of oxygen concentration on embryo quality and implantation rate, the patients were randomly divided into two groups in accordance with gas phases, the physiological condition group with 5% O_2, 5% CO_2, and 90% N_2, (n=170) and the atmospheric condition group with 5% CO_2 and air balance (n=205). Embryonic development was assessed on ET day.

3. Embryo Transfer and outcome measurements

Embryo transfers were performed on day 3 after oocyte retrieval. The quality of embryos was graded on the basis of our grading criteria. Briefly, the grade I represented the best quality. Embryos of grade I, I-1, and II were considered to be 'good' embryos, and grade II-1 and III were regarded as 'poor' quality embryos. Patients were monitored by beta-human chorionic gonadotropin (β-hCG) levels in serum 11-14 days after ET. If the β-hCG level is greater than 5mIU/ml, the patient was considered 'chemical pregnant and repeated the serum test two days later. Diagnosis of clinical pregnancy was performed by detection of gestational sac in sonography 5 - 6 weeks after ET.

4. Statistical Analysis

Results are expressed as mean ± standard deviation. The chi-squared test or t-test was used for statistical analysis. The p-values were considered statistically significant when $p<0.05$.

4-3. RESULTS

A total of 375 cycles were analyzed for evaluation of effective oxygen concentration on embryo quality and implantation. There were no significant differences between the physiological and atmospheric condition groups with respect to maternal ages (32.7 ± 3.0 and 32.9 ± 3.1, respectively), average estradiol level on the HCG administration day (2332.3 ± 1180.3 and 2387.2 ± 1247.1, respectively), oocyte maturation rate (82.9% and 82.8%, respectively), and fertilization rate (72.9% and 72.5%, respectively). The good embryo rate showed no significant difference between the physiological condition group (62.5%) and the physiological condition group (58.2%) (Table 4-1).

However, the implantation rate was significantly higher in the physiological condition group (23.4%) than the atmospheric condition group (18.4%). Clinical pregnancy and delivery rates showed no significant difference between the physiological condition group (44.1% and 37.6%, respectively) and the atmospheric condition group (38.8% and 29.8%, respectively) (Table 4-2).

Table 4-1. Characteristics of the two study groups cultured under either 5% or 20% oxygen concentration

	5% O_2 [b]	20% O_2 [b]	*p*-value
No. of IVF-ET cycles	170	205	
No. of patients	168	198	
Mean of female age (year)[a]	32.7 ± 3.0	32.9 ± 3.1	NS
Estradiol concentration (pg/㎖)[a]	2332.3 ± 1180.3	2387.2 ± 1247.1	NS
No. of retrieved oocytes[a]	15.9 ± 7.8	16.2 ± 8.4	NS
Oocyte maturation rate (%)	2240/2703 (82.9)	2743/3313 (82.8)	NS
Fertilization rate (%)	1734/2380 (72.9)	2093/2887 (72.5)	NS
Good embryo rate (%)	776/1241 (62.5)	870/1494 (58.2)	NS
No. of transferred embryo[a]	576 (3.4 ± 0.8)	690 (3.4 ± 0.8)	NS

[a] Note: values are Mean ± SD.

[b] The concentrations of 5% O_2 and 20% O_2 represent the atmospheric and physiological conditions, respectively.

NS represents ‘not significant’

Table 4-2. Comparison of clinical pregnancy, implantation, and delivery outcomes between 5% O_2 and 20% O_2 groups

	5% O_2 [a]	20% O_2 [a]	*p*-value
No. of IVF-ET cycles	170	205	
Clinical pregnancy (%)	75 (44.1)	77 (37.6)	NS
Implantation rate (%)	135/576 (23.4)	127/690 (18.4)	0.0331
Delivery rate (%)	66 (38.8)	61 (29.8)	NS

[a] The concentrations of 20% O_2 and 5% O_2 represent the atmospheric and physiological conditions, respectively.

NS represents ‘not significant’.

4-4. Discussion

Incubators are one of the most important equipment in IVF laboratories for successful embryo culture. Thanks to technological advances, different types of incubators have been developed to regulate environmental variables such as, gas concentrations, humidity and temperature (Swan, 2014). There are a variety of factors that affect IVF results, other than the culture condition related to incubator types. One of the conditions that have the greatest effect on the IVF results could be an oxygen concentration, because the air atmospheric condition is higher than the physiological oxygen condition of the female reproductive tract that has 2 to 8% oxygen in it. The high oxygen concentration (20%) can be detrimental to embryo development and gene expression as shown in some studies in the model of mice and cattle (Wale and Gardner, 2012). While, the 5% oxygen concentration has been found benefit for both animal and human embryo development and pregnancy outcomes (Meintjes et al., 2009; Kasteretein et al., 2013). However, these results contrast with other previous studies that showed no benefit in culturing human embryos at lower concentrations of oxygen (Dumoulin et al., 1995, 1999). There have still been controversies in the effects of oxygen on success of human IVF.

It was revealed that the fertilization rates and embryo quality were similar between 5% and 20% oxygen concentrations. These results may be similar to Guo's study (2014) that showed no difference in the fertilization and cleavage rates in the first two cleavage divisions. But, embryos of 5% oxygen concentration group had higher percentage of blastocyst formation rate than the 20% oxygen concentration group (64.5% vs. 52.9%, P= 0.009). It would be due to sub-optimal culture condition in vitro, especially exposure to 20% oxygen concentration has association with increased ROS generation and compromised developmental ability (Guo et al. 2014). In addition, Wale et al. (2010) showed that the zygotes stage was most sensitive to oxygen toxicity. In this study, exposure of mouse embryos to high oxygen concentration resulted in delay in cleavage division. Bredaiwy et al. (2004) reported that high ROS levels on day 1 in the culture media were associated with low fertilization rate, low cleavage rate, and high embryonic fragmentation during ICSI cycles. Like many other studies, our study also concluded that physiological oxygen concentration was better to embryo development.

Although, there were no significant differences in IVF results including fertilization, good embryo clinical pregnancy, implantation rate of 5% oxygen concentration group was significantly higher than that of 20% oxygen concentration group. I also demonstrated that 5% oxygen concentration was more effective on implantation rate than 20% oxygen concentration.

Chapter 5

Comparison of Embryo Quality and Pregnancy Rate According to the Types of Incubator

5-1. INTRODUCTION

Multiple incubator types exist with various capabilities and different methods of regulating their internal environment (Higdon et al., 2008; Swain, 2014). Standard large box-type incubators (SI), initially developed to hold multiple flasks of somatic cells, have been long used for clinical IVF (Ham et al., 1962). They used thermal conductivity (TC) CO_2 sensor which are impacted by temperature and humidity. The humidity is provided via evaporation from a pan of water placed on the bottom of the incubator. But, the presence of a water pan is a potential source of microbial contamination, which can negatively affect the embryo development. Recently, a variety of moisture-free benchtop incubators (BTI) outfitted with infrared (IR) sensors have been specifically developed for embryo culture (Swan, 2017). They provide a faster CO_2 recovery compared to the incubator with TC CO_2 sensor following door opening and consist of several small chambers with the heated surface which allows contact to the dish surface directly (Fujiwara et al., 2007). Furthermore, these incubators the use of the cylinder of premixed gas in the BTI may provide improvement in air quality over use of ~94% room air in the SI. Appropriate CO_2 and O_2 concentrations are quickly achieved as soon as the incubator volume has been filled with the premixed gas.

Some studies have been carried out comparing human embryo development and clinical outcomes between BTI and SI, clinical outcomes in humans are controversial. No significant differences were found in blastocyst formation, quality and ongoing pregnancy of embryos cultivated either BTI or SI (Cruz et al., 2011). In spite of reduction in environmental changes caused by frequent door open and close in the time-lapse systems, no significant differences were found in clinical pregnancy rate or implantation rate of embryos cultivated between the time-lapse imaging incubator and SI (Cruz et al., 2011; Kirkegaard et al., 2012; Park et al., 2015). On the other hand, Fawzy et al. (2017) have recently reported that human embryos cultivated ex vivo in a BTI showed significantly decreased implantation rates and clinical and ongoing pregnancy rates. They said the in vivo condition is humid, and mimicking this in vitro seems to be physiologically sound and there is no universal agreement among scientist regarding the advantages of SI versus BTI. Another some studies showed an increase in clinical pregnancy rate of embryos grown in the benchtop/time-lapse incubator compared to the large-box CO_2 incubator with a TC sensor (Mesebuer et al., 2012). This study was aimed to compare the development and clinical outcome according to the incubator type, BTI and SI.

5-2. MATERIALS AND METHODS

1. Patients, controlled ovarian hyperstimulation, and oocyte retrieval

Total 438 ICSI cycles from 414 patients were analyzed in this study – SI incubator (n=230) and BTI incubator (n=208). Only the cycles in which 5 or more oocytes were retrieved followed by ET on day 3 were included in this study. When the female age was more than 40 years old, the cycles was not included in this study. Donor cycles and preimplantation genetic diagnosis (PGD) cycles were also excluded.

Ovarian stimulation was carried out using gonadotropin releasing hormone (GnRH) agonist/antagonist, human menopausal gonadotropin (hMG), and human recombinant follicle stimulating hormone (FSH). Human chorionic gonadotropin (hCG) was administered when optimal follicle development was achieved, as evaluated by serial transvaginal ultrasound and estrogen determinations. Oocyte retrieval was performed via a transvaginal approach with sonographic guidance 34 hours after hCG injection.

2. Sperm preparation, in vitro fertilization and embryo culture

Semen samples were collected immediately after oocyte pick-up. Sperm concentration and motility were analyzed by a light microscopy (BI41T, OLYMPUS, Japan). Sperm was prepared either by swim-up method or by the density gradient centrifugation method. In conventional IVF, the oocytes allocated to IVF were maintained in fertilization medium until the sperm samples were prepared. The oocyte–cumulus complexes were inseminated with 50,000 or more motile spermatozoa per insemination dish. After 16-18 hour, the inseminated oocytes were stripped from the cumulus cells to check for fertilization and the maturity status of the non-fertilized. For ICSI cycles, cumulus cells were removed approximately 1 hour after oocyte retrieval using 80IU/mL of hyaluronidase. The remaining cells were mechanically removed by gentle pipetting with glass capillaries. Among the oocytes allocated to ICSI, mature metaphase II oocytes were selected and injected with sperm. ICSI was performed at least 1 hour after removing the cumulus cells. Fertilization was assessed 16 hours after ICSI. Only oocytes with two pronuclei and the second polar body were assessed as normally fertilized oocytes. The zygotes were cultured in cleavage medium in either SI (Heracell 150i, Thermo Scientific) or BTI (G185, K-SYSTEMS) under 37°C, 6% CO_2, and 5% O_2. Embryonic development was assessed on Day 3 after insemination.

3. Embryo Transfer and outcome measurements

Embryo transfers were performed on day 3 after oocyte retrieval. The quality of embryos was graded on the basis of our grading criteria. Briefly, the grade I embryos, even blastomeres and no fragmentation; grade I-1 embryos, even blastomeres and <25% fragmentation; grade II embryos, uneven blastomeres and <25% fragmentation; grade II-1 embryos, uneven blastomeres and 25% to 50% fragmentation; and grade III embryos, even or uneven blastomeres and ≥50% fragmentation. The grade I represented the best quality. Embryos of grade I, I-1, and II were considered to be ‘good’ embryos, and grade II-1 and III were regarded as ‘poor’ quality embryos. Patients were monitored by beta-human chorionic gonadotropin (β-hCG) levels in serum 11-14 days after ET. If the β-hCG level was greater than 5mIU/ml, the patient was considered ‘chemical pregnant and repeated the serum test again two days later. When a gestational sac was detected in sonography 5 - 6 weeks after ET, it was diagnosed as clinical pregnancy.

4. Statistical Analysis

Results were expressed as mean ± standard deviation (SD). The chi-squared test or t-test was used for statistical analysis. The p-values were considered statistically significant when $p<0.05$.

5-3. RESULTS

In this study, 438 patients met the inclusion criteria. Of these, 3,023 oocytes from 230 patients were culture in SI and 2,125 oocytes from 208 patients were cultured in BTI for 3 days. All baseline characteristics were similar between the SI and BTI groups except for number of matured oocytes (Table 5-1). The mean number of metaphase II oocytes was higher in the SI group than the BTI group ($p < 0.05$). The causes of infertility did not differ between the groups (Table 5-2).

The fertilization rate per oocyte retrieved was not different between two groups. Significantly more zygotes were cultured in the SI groups when compared to the BTI (SI: 6.7 ± 2.7 and BTI: 5.4 ± 2.3, $p < 0.05$). The good embryo rate of the SI group was significantly lower than that of the BTI groups (SI: 77.8% and BTI: 69.2%, $p<0.05$), but there was no significant difference in transferred good embryo rates (SI: 93.4% and BTI: 92.8%). The mean number of transferred embryo (SI: 2.5 ± 0.6 and BTI: 2.4 ± 0.6) and the β-hCG positive rates (SI: 52.6% and BTI: 52.9%) showed no significant difference between the SI and BTI groups. Likewise, there was no significant difference in clinical pregnancy rates between SI and BTI groups (40.9% versus 41.3%) (Table 5-3).

Table 5-1. Characteristics of the two study groups cultured in either Standard large box-type incubator or Benchtop incubator

	Standard large box-type incubator	Benchtop incubator	*p*-value
No. of ET cycles	230	208	
Mean of female age (year)[a]	35.3 ± 3.3	35.9 ± 3.7	NS
No. of retrieved oocytes[a]	13.1 ± 6.6	10.2 ± 4.8	< 0.05
Oocyte maturation rate (%)[a]	75.2 ± 0.4	76.0 ± 0.3	NS

[a]Note: values are Mean ± SD.

NS represents ‘not significant’.

Table 5-2. Causes of infertility of the two groups cultured in either Standard large box-type incubator or Benchtop incubator

	Standard large box-type incubator	Benchtop incubator	*p*-value
No. of ET cycles	230	208	
Male factor (%)	30.3	26.9	NS
Female factor (%)	21.7	27.4	NS
Combined factor (%)	33.0	33.7	NS
Unexplained factor (%)	15.2	12.0	NS

NS represents ‘not significant’.

Table 5-3. Comparison of embryo development and implantation rates between the two groups

	Standard large box-type incubator	Benchtop incubator	*p*-value
No. of ET cycles	230	208	
Fertilization rate (%)	1799/2532 (71.1)	1234/1693 (72.9)	NS
Mean No. of cultured embryo[a]	6.7 ± 2.7	5.4 ± 2.3	< 0.05
Good embryo rate (%)	1062/1534 (69.2)	867/1114 (77.8)	< 0.05
ET good embryo rate (%)	535/573 (93.4)	465/501 (92.8)	NS
Mean number of transferred embryo[a]	2.5±0.6	2.4±0.6	NS
β-hCG positive rate (%)	121 (52.6)	110 (52.9)	NS
Implantation rate (%)	122/573 (21.3)	109/501 (21.8)	NS
Clinical pregnancy rate (%)	94 (40.9)	86 (41.3)	NS

[a]Note: values are Mean ± SD.

NS represents ‘not significant’.

5-4. Discussion

Incubators are one of the most important equipment in IVF laboratories for successful embryo culture in vitro. Thanks to technological advances, different types of incubators have been developed to regulate environmental variables such as, gas concentrations and temperature (Swan, 2014). Among the newly developed incubators, BTI seems to be receiving the most attention in these days because BTI can prevent evaporation of culture medium only by covering it with oil, and more direct temperature control to culture dish, while SI system requires humidity with a water tray inside of the incubation chamber and more indirect temperature control depend on the purpose. Sometimes, water in the tray is contaminated causing total contamination of oocyte and/or embryos. Therefore, BTI has been considered highly effective in IVF procedure to prevent such contamination by humidifying vapor.

However, there has been controversial result in using BTI and SI system. Except humidification system, there are several major differences between them including interior chamber size, temperature control, gas phase, recovery time and gas delivery method (Fujiwara et al., 2007; Swan, 2014). The BTI consists of several small chambers to fit exact height of the culture dish and supports direct heat transfer to the culture dish by lower heated surfaces. Appropriate CO_2/O_2 concentrations also can be quickly achieved via premixed gas system of BTI following door opening. With these properties, Fujiwara et al. (2007) have previously reported that oxygen concentration was more rapidly recovered in BTI than conventional incubators, which influenced increase of early embryo and blastocyst development. On the contrary, Fawzy et al. (2017) have recently reported that human embryos cultivated ex vivo in a dry incubator the kinds of BTI (G185, K-SYSTEMS) had statistically significantly decreased implantation rates and clinical/ongoing pregnancy rates when compare to a humidified incubator such as SI. In this study, the influence of BTI and SI systems was compared on the basis of fertilization rate, embryo quality, clinical pregnancy rates and implantation rates. Our results showed no significant differences between these two groups except embryo quality. The number of good quality embryo in BTI groups was significant higher than that of SI group. This is thought to be due to faster recovery of environmental variables including gas concentrations and temperature in the BTI system.

Lee et al. (2010) also reported that the embryo quality on day 3 after ovum pick up, implantation rates and clinical pregnancy rates did not differ between the Cook-MINC incubator a kind of BTI and the Forma incubator a kind of SI, although the fertilization rate was increased in the Cook K-MINC incubator with significant difference. In addition, other several studies have demonstrated that time-lapse BTI systems showed no significant difference in blastocyst formation rates and quality and/or ongoing pregnancy rates when compared to SI system, despite that the embryos cultured in time-lapse BTI were periodically exposed to light for digital images, possibly causing an unnatural stress to embryos (Cruz et al., 2011; Park et al., 2015). In the more recent retrospective cohort study, culturing and selecting embryos with time-lapse BTI significantly improved the clinical pregnancy rate in comparison with SI, demonstrating that the BTI was superior to the SI (Meseguer et al., 2012). However, this could be due to a variety of factors including, but not limited to, improved embryo selection from time-lapse imaging and not removing embryos for daily observation from the benchtop/time-lapse unit or maintain quality of the incubation.

The reduced O_2 concentration in the culture environment has been found beneficial for both animal and human embryo development and outcomes (Kasterstein et al., 2013; Meintjes et al., 2009). Here, clinical outcomes of embryos cultured between in BTI and SI under low O_2 (5%) and 6% CO_2 were investigated. These results suggest that BTI is more useful for embryo culture in IVF laboratory than SI, because BTI provides faster recovery of environmental variables including gas concentrations and temperature as well as a space-saving size.

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ABSTRACT

Assisted reproductive technologies (ARTs) involve all fertility treatment in which either oocytes or embryos are handled to result in pregnancy, and have been continuously improved since the birth of Louise Brown in 1978 so far. The field of ARTs basically includes *in vitro* fertilization (IVF), embryo culture, and embryo transfer (ET). This study was performed to characterize the factors influencing on embryo quality and outcomes in human ARTs.

In chapter 1, ARTs, such as, intracytoplasmic sperm injection (ICSI), testicular sperm extraction (TESE), blastocyst culture and transfer, culture environment, and incubator were explained and overview of this study was talked.

In chapter 2, efficiency of fertilization methods (conventional IVF and ICSI) was compared. Intracytoplasmic sperm injection (ICSI) was initially developed to treat male factor infertility. However, recently, ICSI is largely applied even to non-male factor infertility, without solid evidence of effectiveness of ICSI in those cases. Thus, this study has compared the outcomes of conventional IVF and ICSI with split-insemination method, and confirmed the efficiency of ICSI on non-male factor infertility. The fertilization rate and embryo quality were not different between IVF and ICSI in split insemination cycles. According to results, ICSI in non-male factor infertility was unnecessary.

In chapter 3, to analyze the efficacy of testicular sperm, this study focused on

testicular sperm extraction (TESE) technique. ICSI combined with TESE (one of the most innovative and highly contributing ARTs) has been successfully established now, and reported to achieve good fertilization and pregnancy outcomes comparable to those of ICSI with ejaculated sperm. In addition, a number of studies have demonstrated that blastocyst transfer could improve the clinical outcomes of IVF. In this study, the clinical outcomes of ICSI performed using ejaculated or TESE sperm were compared in blastocyst ET cycles. There were no significant differences in blastocyst formation and good quality blastocyst rates between ejaculated and TESE sperm groups. Based on these results, blastocyst transfer may be considered when the appropriate numbers of oocyte are retrieved and motile sperm are identified in ICSI cycles using testicular sperm.

In Chapter 4, the culture conditions were evaluated in order to optimize embryo development and to increase the number of good quality embryos. The most important thing in a culture environment is a laboratory incubator and its condition such as oxygen tension, pH, and temperature stability, which can impact on embryo development. Therefore, in this study, the effect of oxygen concentration on embryo quality and implantation rate was examined in IVF-ET cycles. In this study, different gas phases [physiological (5% O_2, 5% CO_2, and 90% N_2) or atmospheric (5% CO_2 with the balance as air) condition] were applied to the retrieved oocytes and embryos during culture periods. There were no significant differences between two groups with respect to fertilization and good quality embryo rates. However, the implantation rate of the physiological (5%) oxygen concentration group was significantly higher than that of atmospheric (20%) oxygen concentration group. Thus, this study could conclude that the physiological oxygen concentration rather than atmospheric one was more effective in increasing IVF success rates, even though fertilization and good quality embryo rates did not reflect the difference of oxygen concentration.

In chapter 5, the effect of incubator types on embryo quality and clinical outcomes was evaluated by comparing two types of incubators, standard large box-type incubators (SI) as a humid type and benchtop incubator (BTI) as a dry type. There were no significant differences in clinical outcomes between two types of incubator except embryo quality. The embryo quality of BTI groups was significant higher than that of SI group. It may be because BTI provides faster recovery of environmental variables including gas concentrations, and temperature.

Numerous ARTs could affect the IVF-ET outcomes directly or indirectly. Therefore, thorough investigation and careful consideration are required when setting up and managing the IVF environment in order to achieve successful results for patients.