Chapter 1

An Overview of Intracytoplasmic Sperm Injection (ICSI): Its Development and Popularity

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Abstract

Assisted reproductive technology (ART) is widely used in infertility treatments to achieve pregnancy. This technology includes in vitro fertilization (IVF) and microinjection (Intracytoplasmic Sperm Injection-ICSI) procedures. Today, ICSI is the most commonly used fertilization method for both severe male factor infertility and non-male-induced infertility in many laboratories all over the world. In this method, a sperm cell is injected into the cytoplasm of an oocyte cell under a microscope with a micromanipulator attached. Although it is a popular method of insemination, it has various indications. Gametes and zygotes are exposed to a non-physiological treatment and culture medium during ART procedures. It is known that this process and environment that they are exposed to can affect the epigenetic properties of male and female gametes and may negatively affect the early embryogenesis process. While most children born with the ICSI method appear healthy,

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there are concerns about the safety and overuse of this technology. When the health status of children born with ICSI is analyzed to date, it has been suggested that children born with ICSI have more congenital malformations, epigenetic disorders, chromosomal anomalies, psychological and neurological developmental disorders than children born naturally. How much of the documented negative effects are connected to parental variables or ICSI, though, is still unknown. In this review, we aimed to discuss the ICSI method, its development and data on epigenetic risks in the light of literature information.

1. Introduction

Infertility has been studied in a variety of aspects including medical, social science, theology, and philosophy. It is described as the inability to conceive after one year of unprotected intercourse and affects around 15% of couples at reproductive age globally (Palermo et al., 2014). For this reason, the use of ART in treating infertile couples has gradually expanded.

The process of Intracytoplasmic Sperm Injection (ICSI) involves the precise injection of a solitary spermatozoon into the cytoplasm of an oocyte, utilising a glass micropipette. The method, which was initially proposed by Palermo et al. in 1992, was intended to enhance the conventional in vitro fertilisation (IVF) technique. Although IVF was initially utilised as the primary approach for fertilisation in assisted reproductive technology (ART) treatments, it was discovered to be inadequate in achieving fertilisation and successful pregnancy. The development of Intracytoplasmic Sperm Injection (ICSI) is considered a significant accomplishment in the realm of Assisted Reproductive Technology (ART), as it enables men with low sperm count and quality, including those with azoospermia, to biologically father a child (Palermo et al., 2015). In contemporary times, Intracytoplasmic Sperm Injection (ICSI) has gained widespread recognition as a laboratory technique employed globally to address issues of infertility. In the realm of assisted reproductive technology, intracytoplasmic sperm injection (ICSI) was originally designed to address the most severe instances of male factor infertility. Nevertheless, it has become increasingly prevalent in cases where male factor infertility is absent, despite the absence of definitive proof of its superiority over conventional in vitro fertilisation (IVF) (Van Rumste et al., 2004; Dyer et al., 2016). The first fertilization obtained by the ICSI method was announced by Lanzerdorf et al. in 1988 in humans, but pregnancies and live births in humans by the ICSI method were first reported in 1992 by Palermo et al. (Lanzerdorf, 1988; Palermo et al., 1992). Since this date, the ICSI method has been the main method used to ensure fertilization in

individuals who cannot conceive naturally (Van Steirteghem et al., 2002). In addition to ICSI applications in humans, ICSI applications have been made in many animal species. For the first time, successful ICSI application was obtained in rabbits in 1989, in cattle in 1990, in sheeps in 1996, in horses in 1998 and in dogs in 1998 (Iritani, 1989; Goto et al., 1990; Catt et al., 1996; Cochran et al., 1998; Fulton et al., 1998). Upon reporting the birth of a baby using the ICSI method (Palermo et al., 1992), this method has gained popularity in other European countries and its use has become widespread worldwide.

Throughout the 1990s, the therapeutic applications of intracytoplasmic sperm injection (ICSI) have encompassed a range of procedures, including the utilisation of spermatozoa exhibiting weak progressive motility, as well as the microsurgical retrieval of gametes from the epididymis and testis of azoospermic patients (Palermo et al., 1995; Palermo et al., 1999; Wallach et al., 1996). To date, more than two million babies have been born worldwide thanks to ICSI (ESHRE, 2012; Sullivan et al., 2013) and have been successfully used to bypass zona pellucida irregularities, antisperm antibodies, sperm acrosome dysfunction, and sperm kinetic defects, using previous assisted insemination techniques (Palermo et al., 1996).

The use of ICSI method has been reported to be beneficial for the fertilization of cryopreserved oocytes since cryostress can prevent spontaneous entry of spermatozoa by causing premature exocytosis of cortical granules and zona hardening (Johnson, 1989; Schalkoff et al., 1989; Vincent et al., 1990; Van Blerkom and Davis, 1994; Porcu et al., 1997). ICSI is often the preferred method of insemination to avoid polyspermia, fertilizing large numbers of oocytes and producing large number of embryos. Also, using a single sperm minimizes the risk of HIV, HBV and HCV transmission. The presence of virus in sperm or accompanying cells can be reduced by removing seminal fluid with density gradient preparations and directly removing sperm cells from a viscous medium just before injection (Vitorino et al., 2011), thus ICSI has become the method of choice for insemination of HIV-infected patients (Pena et al., 2002; Sauer and Chang, 2002; Mencaglia et al., 2005). ICSI is also not directly affected by immature sperm from the epididymis/testis, and cells in this part often have an incomplete flagellum and an immature cell membrane (Palermo et al., 1996; Palermo et al., 1999). Successful pregnancies using these spermatozoa have extended the applicability of ICSI to the extremes of male infertility, such as cryptozoospermia, virtual azoospermia, or absolute azoospermia requiring surgical intervention (Ron-El et al., 1997).

ICSI has emerged as the most popular fertilisation technique used in ART as a result of these features. ICSI's high level of standardisation and validation, which allowed fertility clinics all over the world to quickly adopt the technique into routine practise, and the potential for its use in treating nearly all causes of infertility are potential explanations for its widespread use (Palermo et al., 2015). According to Adamson et al. (2018), 66.5% of fertility clinics employ the ICSI procedure today. ART data reported on the US National Assisted Reproductive Technology Surveillance System between 1996 and 2012 was examined by Boulet et al. They reported that ICSI was employed in 65.1% of all fresh IVF cycles (embryos transplanted without freezing; n = 1,395,634) in accordance with the data they had collected (Boulet et al., 2015). Although there was no change in approximately 36% of cases as a result of the application of ICSI as a fertilization method in male factor infertility, the use of ICSI increased from 36.4% in 1996 to 76.2% in 2012 (Boulet et al., 2015). Dyer et al. evaluated worldwide ART data generated by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) between 2008 and 2010. Approximately 67% of the 4,461,309 ART cycles analyzed were found to be fertilized by ICSI (Dyer et al., 2016). There is a great variation between geographical regions in terms of ICSI use. ICSI is performed in approximately 55% of ART procedures in Asia, 65% of cases in Europe, 85% of cases in Latin America, and almost 100% of cases in the Middle East (Dyer et al., 2016). The use of ICSI has increased significantly due to its reliability in ensuring fertilization in severe male factor infertility cases.

In conjunction with preimplantation genetic testing (PGT), ICSI is increasingly frequently used for patients with advanced maternal age, cryopreserved oocytes, and women with low oocyte maturation (Adamson et al., 2018). Preimplantation genetic testing (PGT) is a process that patients prefer to have done in order to assess the genetic profile of embryos, delay conception, and increase the likelihood of implantation and a successful pregnancy (SART, 2017). All of the aforementioned elements have helped ICSI become more popular. There is some disagreement over whether ICSI is superior to IVF despite its rising popularity. When the effect of the ICSI method on pregnancy rates and birth rates was analyzed, pregnancy rates were 28.7% and birth rates were 18.9% in 2008, pregnancy rates were 27.7% and birth rates 19.9% in 2009, pregnancy rates were reported as 26.8% and birth rates as 20.0% in 2010 (Dyer et al., 2016). In line with these reported analyses, it can be said that pregnancy rates and birth rates with the ICSI method remain relatively low. It has been reported that ICSI is associated with lower implantation rates and lower live birth rates in cases

of non-male infertility when patients who underwent classical IVF and ICSI method were compared (Boulet et al., 2015). Chambers et al. revealed that ICSI had no effect over conventional IVF in couples with non-male infertility in a large retrospective cohort research analyzing 585,065 ART cycles in Australia between 2002 and 2013. In contrast, the adjusted odds of live birth was reported to be approximately 10% lower with ICSI than with IVF (Chambers et al., 2016). In line with these results, the Practice Committee of the American Society for Reproductive Medicine (ASRM) recommended not to use ICSI in cases of non-male infertility (Practice Committees of American Society for Reproductive Medicine and Society, 2012). Concerns have also been raised concerning whether the indiscriminate use of ICSI results in negative health outcomes for the offspring (Boulet et al., 2015). Upon reporting the birth of a baby using the ICSI method (Palermo et al., 1992), this method gained popularity in other European countries and its use became widespread worldwide.

ICSI became the most widely used ART in the twenty-first century, but how? In this study, we discuss the early micromanipulation methods that contributed to the development of ICSI and its later broad application. We will discuss the surge in its popularity as well as possible future uses for this versatile fertilisation technique. In this review, we provide an overview of the available data on the use of ICSI with a particular emphasis on male factor infertility. We provide a historical overview of the development of ICSI, inform about its applications for cases of male factor and non-male factor infertility, and describe the specific technical aspects of ICSI when used to treat male factor infertility. We also provide and critically evaluate the evidence on ICSI's effects on infants health.

2. Methods Used Before ICSI

Data regarding IVF as a viable alternative for infertile couples emerged in 1978 when a woman with bilateral tubal occlusion had the first live birth (Steptoe and Edwards, 1976; Steptoe and Edwards, 1978). This was the first pregnancy achieved with natural cycle IVF without the assistance of ovarian superovulation (Wang and Sauer, 2006). Unstimulated cycles were utilized by all doctors until 1982, with a pregnancy rate of 6% obtained. Researchers reported an average of 2.1–2.6 oocytes per harvest and a clinical pregnancy rate of 30% in a trial employing human menopausal gonadotropin (hMG) and enhanced oocyte counts (Edwards and Steptoe, 1983). The gamete intrafallopian transfer operation, one of many attempts to increase conception chances, involves injecting male gametes directly into the fallopian tubes. In this procedure, spermatozoa and oocytes were

transferred to the fallopian tube laparoscopically (Asch et al., 1984). Because it was not advised for female patients with tubal obstruction or male patients with aberrant semen parameters, this practise was quickly phased out. Later, a novel version of this technique known as the zygote intrafallopian transfer (ZIFT) approach was presented. In this method, the oocyte is fertilised in vitro before being transferred to the fallopian tube. The advantages of this method outweigh the disadvantages of prior methods. However, because oocyte retrieval and zygote transfer require two separate laparoscopic procedures, this method has been found to be invasive, unsuccessful, and costly (Hamori et al., 1988). This became especially clear when transvaginal oocyte aspiration, a less invasive procedure, became commonplace. However, these patients unintentionally started to suffer from fertilisation failure. Later, a problem with the semen parameters was found to be related to this. Traditional IVF was effective in treating tubal infertility and was soon modified to help couples who had male partners who exhibited abnormal kinetic or morphological features (Cohen et al., 1985; Cohen et al., 1989). In microdroplet insemination, the use of pooled oocytes and tiny oil-coated droplets filled with concentrated sperm was suggested (Svalander et al., 1994), however the fertilisation rate was still subpar and unpredictable. Numerous methods have been tried to enhance fertilisation results, with the first method concentrating on zona pellucida (ZP) manipulation. Because it was believed at the time that the spermatozoa wouldn't be able to penetrate the thick glycoprotein covering of the egg (Palermo and Rosenwaks, 1995). In order to help the male gamete and oolemma unite, it was decided to fully eradicate ZP in an early experiment. However, this led to a high percentage of polyspermy. This method has been hindered by lower embryo development dynamics as a result of the absence of ZP as a framework for constructing concepts (Yanagimachi, 1984). Researchers considered using trypsin or pronase to thin the ZP as a solution to this issue (Kiessling et al., 1988). In patients who had previously had unsuccessful fertilisation, sperm penetration was still present, but the powerful enzymatic action prevented the embryos from lysing (Kiessling et al., 1988; Gordon et al., 1988). Through the use of a tiny glass pipette and acidified Tyrode media, Gordon and Talansky invented a procedure known as zona drilling (ZD), which enables spermatozoa to bypass the zona plate and come into direct contact with the oolemma. Despite the fact that the fertilisation rate increased to 32%, a considerable rate of oocyte damage was produced by the applied acidic environment. Additionally, the polyspermy rate rose as more spermatozoa were able to simultaneously access the perivitelline region via the fissure in the zona (Gordon and Talansky, 1986; Palermo and Rosenwaks, 1995).

The zona pellucida (ZP) is a thin, amorphous, glycoprotein-rich membrane that surrounds the oocyte. Its main functions are to enable sperm to recognize the oocyte thanks to the receptors it contains, to allow fertilization, to prevent fertilization of the oocyte with more than one sperm, and to protect the early embryo from external effects. Spermatozoons show an acrosome reaction to pass the ZP structure and enter the cell by melting the outer layer of ZP. However, in some individuals, due to problems such as oligozoospermia, the sperm cannot pass the ZP and infertility problems occur. In this case, alternative methods have emerged that can pass a small number of spermatozoa into or behind the zona. They are divided into 3 parts as dissolution of the zona pellucida, cutting of a part of the zona pellucida and subzonal sperm injection (SUZI). These methods are described in sub-headings below.

2.1. Melting the ZP Structure

It is used by preparing an acidic solution to dissolve ZP. The prepared solution is drawn onto the micropipette and sprayed onto the ZP with the help of a manipulator pipette on the oocyte, which is held with the holder pipette. In this way, the acid dissolves the ZP structure and creates a hole there **(Figure 1)**.

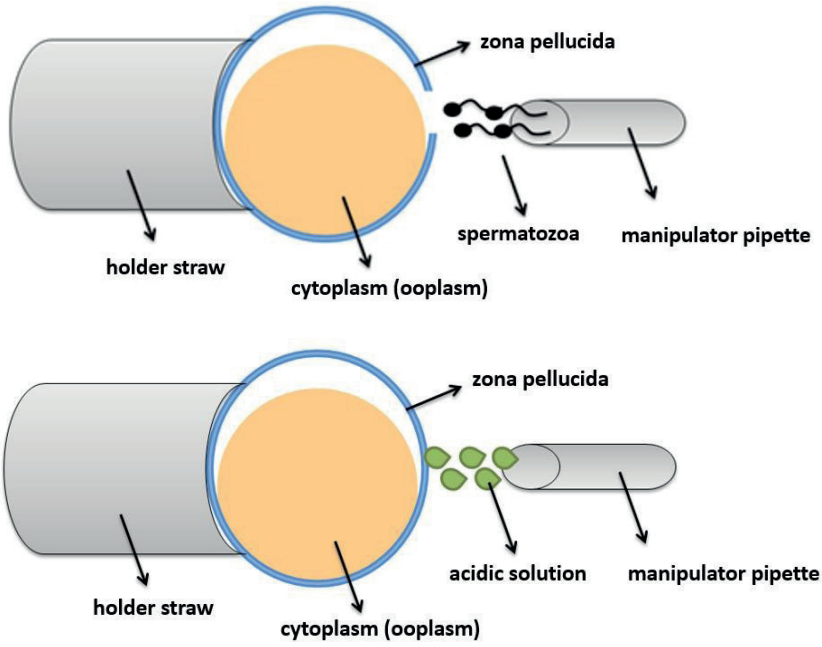

Figure 1. Shingles lysis and spermatozoa release.

2.2. Cutting off Part of ZP

This method is a more controlled method than the puncture method of the zona pellucida. In this method, the oocyte holder is held with a pipette and the ZP is punctured on one side with a pointed manipulator pipette. The manipulator pipette is advanced through the oocyte and removed from the other side, and the oocyte is released with the holding pipette. A 25-40 μm hole is opened in the ZP of the oocyte by rubbing the manipulator pipette holder pipette that enters from one end of the oocyte and exits from the other. This technology is an old technology and nowadays laser cutting and drilling are also used. This method facilitates the entry of spermatozoa into the oocyte and the exit of the resulting embryos from the zona **(Figure 2).**

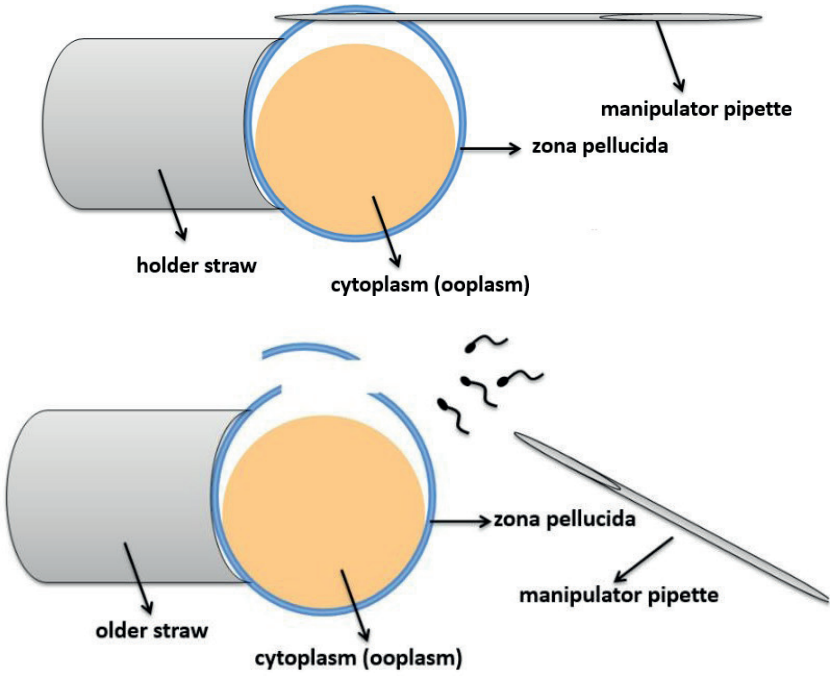

Figure 2. Removal of part of the zona pellucida and removal of spermatozoa

2.3. Subzonal Sperm Injection (SUZI)

This method, which is safer than piercing or cutting the zona pellucida, is also used to control the polyspermia condition. The retained oocyte is entered with a manipulator pipette. One or more spermatozoa are deposited in the perivitelline space. This number may vary depending on the type

of creature. Although Palermo et al. (Palermo and Van Steirteghem, 1991) obtained successful results when they left a single spermatozoa with acrosome reaction in the perivitelline space in their study in mice, since it is difficult to create an acrosomal reaction in human semen, more than one spermatozoa is left in the perivitelline space for the purpose of fertilization of one of them. Thanks to this method, the time required for the penetration of spermatozoa into the perivitelline space is considerably shortened. Although polyspermia can be observed in ten or more spermatozoa, leaving an average of 4-6 spermatozoa is considered the most ideal method in humans (Yamada et al., 1988; Catt et al., 1994; Catt, 1996) **(Figure 3)**.

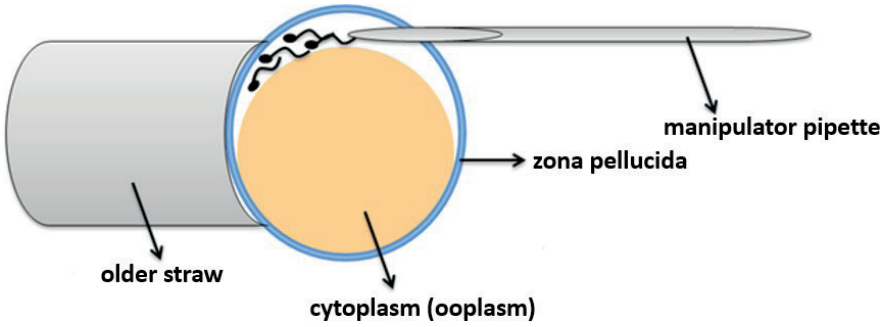

Figure 3. Subzonal Sperm Injection

Since the various fertilization techniques described above both cause polyspermia and similar problems and cause low fertility rates, scientists have focused on an alternative method called "Intracytoplasmic Sperm Injection (ICSI)".

3. Micromanipulators

Cells that are too small to be seen by the human eye and are too small to be manipulated by hand. In cases where fertilization could not occur spontaneously, there was a need to apply various manipulations to spermatozoa and oocytes to perform ICSI. Micromanipulator apparatuses are used for these manipulations applied to cells, which are robust, easily movable and can easily perform the desired intervention without damaging biological materials, and can be connected to microscopes.

Micromanipulators are devices that reduce macro-scale movements to micro-dimensions **(Figure 4)**. In other words, they reduce centimeter-sized movements to millimeters and micrometers. These movements take place via micropipette and micropipette holder microtools, which connect the control

units to the movement units. ICSI requires holding and injection pipettes. These pipettes are attached to the microinstruments, and the microtools to the motion units. Before manipulations begin, the microinstruments are placed and aligned for easy manipulations. These alignments before starting are called the starting position. In practical applications, while the petri dishes and solutions to be reused after the first ICSI process is completed, micro-instruments and micropipettes should be brought to the starting position. To achieve this, movable manipulators are used. These manipulators provide centimeter-level movement, either mechanically or electrically.

Figure 4: Micromanipulator

The advantage of mechanical manipulators is that they provide ease of processing without creating cable density in the laboratory. However, it is very important to adjust the arms of the manipulator before each operation. On the other hand, the biggest advantage of electrical manipulators is that they have control levers in the form of joysticks right next to the microscope. This allows easy operation without losing eye contact with the cells viewed under the microscope in all different types of manipulations to be made.

Magnifications of 200X to 400X are often used for manipulation of the human oocyte cell. Spermatozoons are cells much smaller than oocytes. A very stable and robust system is needed to manipulate cells of this size without vibration and damage. In order to provide these desired features, a

control panel, motion system and micro-instruments are used that transfer the movement of the manipulator to the micropipettes, which can be mounted on the right and left sides of the microscope table, depending on the type of microscope and micromanipulator. There are two main types of manipulators that are widely used in the world, the electronic manipulator and the hydraulic manipulator. These structures have different features in the electronic manipulator and different in the hydraulic manipulator.

3.1. Hydraulic Micromanipulators

ICSI applications require very sensitive manipulations. The most important manipulator that can provide these manipulations is hydraulic manipulators. Similar to the electronic manipulator, the most important advantage of this manipulator is that the microscope and other operating arms are located on the side of the microscope, thus preventing excessive movements that may occur during various adjustments or manipulation.

The hydraulic manipulator is structurally divided into 3 parts: the control unit, the movement unit and the micro-instruments to which the micropipettes are attached.

3.1.1 Hydraulic Manipulator Control Unit

The control unit can be placed close to the microscope. They are fixed to a metal table used to hold on to the table with the magnetic part on their base. In this way, the manipulator, which is fixed so that it does not move, allows easy maneuvers thanks to the control bar. There is 1 knob under the control bar and 2 knobs above it. These knobs give a 3-dimensional mobility in the X, Y and Z plane to perform different manipulations on the oocyte, embryo or spermatozoon **(Figure 5)**. Although it works with a hydraulic oil system, there is no pressure force on the control units in this system. While the movement speed of the manipulator pipette is adjusted by the movement speed adjusting ring in the control unit, the pipette precision is adjusted by the pressure adjusting ring.

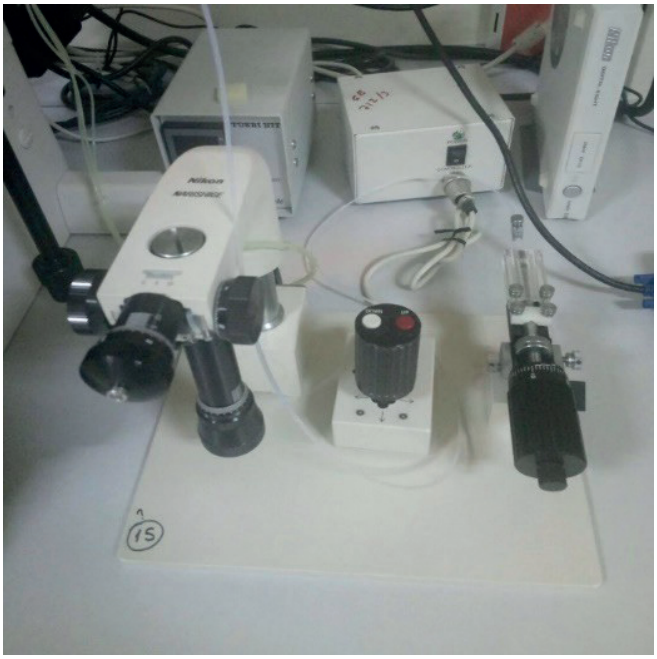

Figure 5: Hydraulic micromanipulator control panel

3.1.2. Hydraulic Manipulator Motion Unit

The second part of the hydraulic manipulator is the movement part. This unit, which is placed on the right and left of the microscope, is responsible for fulfilling the order received from the control panel in a 3-dimensional way **(Figure 6)**. In the systems used in the past, the order received on the control panel is transferred to the motion unit by means of 3 hydraulic pipes. Thanks to these tubes, the motion unit implements the command from the control unit and moves the micro-instruments to which the micropipettes are attached. In the hydraulic manipulator, this part performs its movement mechanically through the hydraulic fluid coming from the hydraulic pipes (Joris, 2012).

Figure 6. Movement unit connected to the right and left of the micromanipulator.

Unlike the old type hydraulic micromanipulator movement unit described above, new type micromanipulators provide convenience in terms of installation and use. In the new type of hydraulic micromanipulators, the micropipette is connected to the motion unit with a single apparatus. The drive unit is in the pulled-out position when not in use. When it will be used, it can be brought to the appropriate position thanks to the positioner button. Angle adjustment screw connected to the motion unit is used to adjust the angles of the micropipettes, and there is a protractor to calculate the same angle in later applications **(Figure 7).**

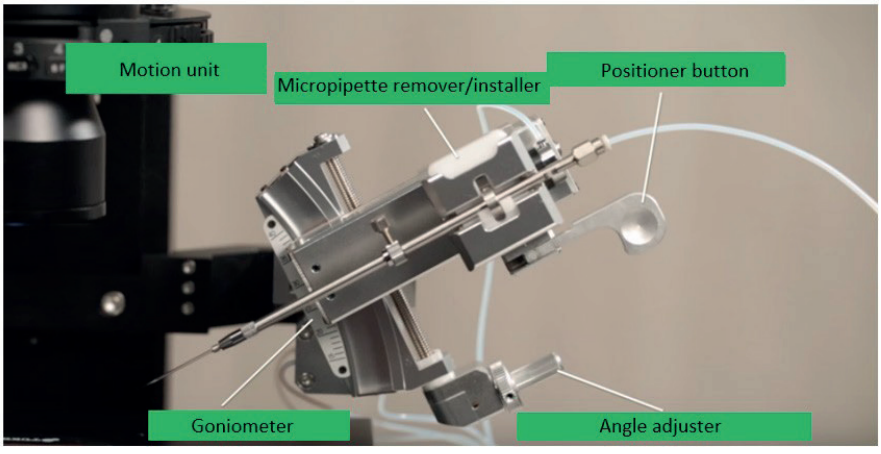

Figure 7: New type of micropipette drive unit.

3.1.3. *Microtools in Hydraulic Manipulator*

It is a set of small-sized instruments found in old-style micropipettes, located between the drive unit and the micropipettes. They can be connected in different positions thanks to the screws on them in order to implement the orders from the movement organs. Connecting the microinstruments in different ways provides different attachment angles to the micropipettes, and thanks to these angles, manipulation of the oocyte and spermatozoa can be done easily **(Figure 8).**

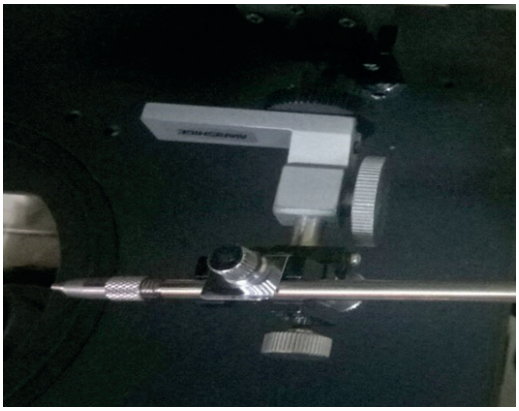

Figure 8: Microtool connector

The positioning of the microtools is very important. The micropipette attached to a properly positioned microinstrument should be positioned parallel to the bottom of the petri dish containing the oocyte. In cases where the positions of the microinstruments are made incorrectly, positions that prevent manipulation arise (Joris, 2012) **(Figure 9).**

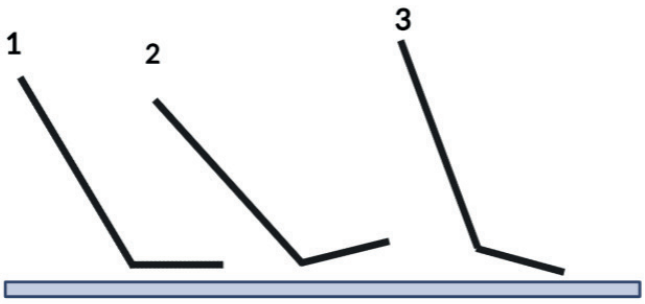

Figure 9: 1 shows the correct position, 2 and 3 show the wrong position. In the second case, the tip of the pipette rises above the oocyte, while in the third case, the pipette is positioned in such a way that it can scratch the bottom of the petri dish and cause a possible blockage.

3.2. Electronic Micromanipulators

Unlike hydraulic manipulators, electronic manipulators are designed entirely electronically. Electronic micromanipulators consist of 2 components, a control unit and a motion unit, since the motion unit and microtools are together.

3.2.1. Control Unit in Electronic Micromanipulators

Control unit; It consists of control bar, display and multi-function keys **(Figure 10)**. The control bar provides the user with planar and vertical movement. Depending on the settings, adjustments such as control position and speed can also be made. In some advanced devices, the previously set position is saved and after the process is finished, it is brought to the first position (or saved position) with the keys on the control bar. Thanks to the control unit, the micropipettes move accurately and effectively manipulate the egg or sperm cell. On the screen part, depending on the operation performed, angles and settings can be adjusted with multi-function keys that can adjust options, and different posture positions can be saved and adjusted.

Figure 10: Electronic control unit

3.2.2. Motion Unit in Electronic Manipulator

In electronic manipulators, the motor (motion) unit can be mounted on either side of any microscope, but to get the best performance, especially in the ICSI procedure, the motor unit should be used by connecting to adapters that are specific to the microscope type and can be mounted on both sides of the microscope. The motor unit consists of 2 main modules connected to each other by wires and screws. These modules have the capacity to make the manipulator move in 3 dimensions in X, Y and Z angles (Abu-Marar and Al-Hasani, 2012) **(Figure 11).**

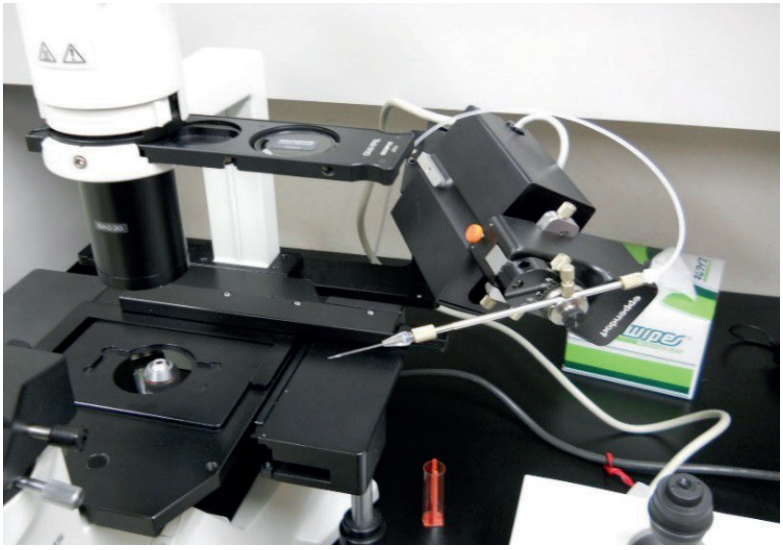

Figure 11: Electronic Micromanipulator Motion Unit

4. Indications for Intracytoplasmic Sperm Injection

Since the birth of Louise Brown in 1978, traditional IVF has been utilised successfully; nevertheless, results are subpar when sperm quantity or quality is insufficient (Palermo et al., 1992). ICSI was initially utilised in cases of male-induced infertility, where the sperm were unable to enter and fertilise an oocyte regularly due to a lack of spermatozoa, poor motility, or an aberrant morphology. Due to the fact that a single sperm with a functional centrosome and genome can fertilise an egg and produce a viable embryo, ICSI has evolved into a natural treatment for couples with severe male-induced infertility (Devroey and Van Steirteghem, 2004). The effects of other reproductive methods, such as the insemination of pre-freeze-stored and in vitro developed oocytes, which block the natural entry of sperm cells

due to the hardening of ZP, have been aded to the list of indications for the use of ICSI.

Male-induced ICSI indications in ejaculation sample include semen parameter abnormalities, structural abnormalities (globozoospermia, primary ciliary dyskinesia), genetic indications (klinefelter syndrome, Yq microdeletions), genomic abnormalities (sperm chromatic fragmentation), infectious agents in semen (HIV/Hep C), ejaculation dysfunction (retrograde ejaculation, electro-ejaculation), presence of other cells and reactive oxygen species and antisperm antibody. Indications from ICSI in surgically retrieved sperm are classified as epididymal (congenital bilateral absence of vas deferens, ejaculatory duct obstruction, post vasectomy, failed vasovasotomy) and testicular (all indication of epididymal sperm, non obstructive azoospermia such as germ cell aplasia, maturational arrest, hypospermatogenesis; postmortem sperm retrieval). Some studies have suggested that the use of ICSI is mandatory in cases of male infertility caused by azoospermia, absolute asthenozoospermia, and globozoospermia (Esteves et al., 2011; Tournaye, 2012; Esteves and Varghese, 2012; Miyaoka and Esteves, 2013; Esteves et al., 2013; Esteves, 2015; Povlsen et al., 2015; Esteves, 2016; Rubino et al., 2016; Dam et al., 2017). High rates of ICSI are also recommended in cases of high sperm DNA fragmentation (Zini et al., 2008; Robinson et al., 2012; Practice Committees of American Society for Reproductive Medicine, 2013; Zhao et al., 2014; Osman et al., 2015; Agarwal et al., 2016; Agarwal et al., 2016; Simon et al., 2017; Esteves et al., 2017) and severe OAT (Tournaye et al., 2002). In the presence of moderate OAT (Shuai et al., 2015), isolated teratozoospermia (Lundin et al., 1997; Osawa et al., 1999; Keegan et al., 2007; Dubey et al., 2008; Hotaling et al., 2011;), and antisperm antibodies (Zini et al., 2011) coupled with a male reproductive tract obstruction, both IVF and ICSI have been shown to be equally effect.

Non-male indications for ICSI include oocyte dysmorphism (zona pellucida abnormalities, oolemma/ooplasmic abnormalities), poor quality oocytes, recurrent polyspermia, in vitro maturation (IVM), advanced maternal age, poor responders, tubal ligation, recurrent polyspermia, fertility preservation (elective oocyte cryopreservation, medically-indicated oocyte cryopresevation) and other factors such as unexplained infertility.

In the case of unexplained infertility, IVF and ICSI are equally effective, but sister oocyte studies show that ICSI is superior to IVF for fertilization, whereas the reproductive outcome is not significantly different (Boulet et al., 2015; Bhattacharya et al., 2001; Johnson et al., 2013; Bungum et al.,

2004; Foong et al., 2006). IVF and ICSI are equally effective in infertility caused by poor quality oocytes, poor responders, and advanced maternal age, but are slightly more in favor of IVF (Ferraretti et al., 2011; Sfontouris et al., 2015; Tannus et al., 2017). While ICSI is highly recommended in preimplantation genetic testing (Thornhill et al., 2005; Harton et al., 2011; Practice Committees of American Society for Reproductive Medicine and Society for Assisted Reproduction Technology, 2012; Babayev et al., 2014), IVF is preferred in infertility caused by tubal ligation.

5. Intracytoplasmic Sperm Injection (ICSI)

ICSI provides significant advantages in terms of performing and controlling the fertilization process without causing polyspermia and shingles damage. In this section, we will examine the ICSI application in all its aspects.

5.1. Preparation of Oocytes

Superovulation is performed prior to oocyte retrieval in order to obtain a larger number of oocytes from patients receiving IVF treatment. Oocytes collected from patients by various methods are transferred to HEPES/CZB (Kimura and Yanagimachi 1995) **(Table 1)**, Whitten's/PVA/HEPES **(Table 2)** or commercial TCM 199 media containing 0.1% hyaluronidase, heated to 37°C. At the end of the oocyte collection process, the tissue residues formed are removed from the medium and within 5 minutes, the cumulus cells begin to separate from the oocyte cells with the effect of the hyaluronidase enzyme. Oocytes separated from cumulus cells are washed with the help of micropipettes by taking hyaluronidase-free HEPES-CZB, Whitten's/HEPES/PVA or TCM 199 drops, and immature, fragmented, abnormal-looking oocytes are eliminated. In the final step, the selected oocytes are placed in a 38.5°C incubator with 5% CO2 in CZB, Whitten's/PVA or TCM 199 medium and left for maturation (Stein and Schultz, 2010; Ward and Yanagimachi, 2018).

Table 1: Advantages and disadvantages of the methods used before ICSI

Method	Place of Release of Spermatozoon	Amount of Spermatozoon Used	Advantages	Disadvantages	Made works
Dissolution of Zona Pellucida	Around the incision or hole created, outside the oocyte	A large number of spermatozoa	-To ensure that spermatozoa can easily overcome the zona pellucida barrier - To facilitate the emergence of the embryo formed after fertilization from the shingles	-The entry of various contamination factors into the cell from the external environment due to the perforation of the zona pellucida - Causes polyspermia, low fertilization data	In the study conducted in mice, 913 oocyte spermatozoa were left with the molten zona pellucida method, 711 fertilizations, 321 blastocysts were obtained, and a success rate of 35% was achieved (Talansky and Gordon, 1988).
Incision of Part of the Zona Pellucida	Around the incision or hole created, outside the oocyte	A large number of spermatozoa	-To ensure that spermatozoa can easily overcome the zona pellucida barrier - To facilitate the emergence of the embryo formed after fertilization from the shingles	-The entry of various contamination factors into the cell from the external environment due to the perforation of the zona pellucida - Causes polyspermia, low fertilization data	In a study conducted in humans, the shingles of 2-5-day-old embryos were cut and the rate of emergence of embryos from shingles was examined. In the control group, 22% of the zona detachment was observed, while it was observed in 53% of the oocytes whose shingles were cut (Dokras et al., 1994).
Subzonal Sperm Injection	Between the zona pellucida and the ooplasm	4-6 spermatozoa	- Possibility of injection without damaging the zona pellucida and without disrupting the integrity of the cell - Reducing the time required for spermatozoa to cross the zona pellucida	Observation of polyspermia when the ideal sperm count cannot be set	In a study comparing Subzonal Injection and In vitro fertilization, it was observed that fertilization was 36% in patients who underwent subzonal injection and 24% in patients who underwent IVF (Fishel et al., 1990).

Table 2: CZB and CZB-HEPES medium

MEDIA CONTENT	CZB	CZB-HEPES
NaCl	4.76 g/L	4.76 g/L
KCl	0.36 g/L	0.36 g/L
KH2PO4	0.16 g/L	0.16 g/L
MgSO4 · 7H2O	0.29 g/L	0.29 g/L
NaHCO3	2.11 g/L	2.11 g/L
CaCl2 · 2H2O	0.25 g/L	0.25 g/L
Na2-EDTA · 2H2O	0.04 g/L	0.04 g/L
L-glutamine	0.15 g/L	0.15 g/L
Na-lactate 60% syrup	3.145 g/L	3.145 g/L
Na-pyruvate	0.03 g/L	0.03 g/L
Glucose	1.00 g/L	1.00 g/L
Penicillin	0.05 g/L	0.05 g/L
Streptomycin	0.07 g/L	0.07 g/L
BSA	4.00 g/L	4.00 g/L
Hepes-Na	---	0.52
NaHCO3	---	0.42
PVA	---	0.10

5.2. Preparation of Pipettes

Two types of pipettes are used to perform ICSI, a holding pipette and a manipulator pipette. Although these pipettes are readily available, in many studies, researchers prepare the pipettes themselves. Manipulator pipettes are responsible for puncturing the zona and releasing spermatozoa into the cell. For this reason, the tip of the manipulator pipettes is prepared in the form of a cone (Acar, 2011). A horizontal pipette puller is used to obtain a properly shaped pipette tip (Horizontal pipette puller, **Figure 12**).

Figure 12. Horizontal pipette puller.

Temperature, time, gravitational force and drafting speed have a direct effect on the formation of the pipette. The horizontal pipette puller applies some heat to the tip of the pipette, and the tip of pipettes, which are mostly made of borosilicate, gains elasticity under the influence of temperature. The pipette puller device pulls the heated pipette tip at a certain speed, thinning it to the desired fineness. The desired fineness level for manipulator pipettes is 5-6 μm on the inner surface of the tip of the pipette and 7-8 μm on the outer surface. Depending on the type of micromanipulator or the user's use, the tip of the pipette is tilted at an angle of 30° to 50°. After the drawing process is done, the tip of the pipette is made conical with the help of a microgrinder when needed according to the intended use of the manipulator pipette. During this process, distilled water or milli-Q water (Palermo et al., 1992) is dripped onto the sharpener to facilitate the sharpening process (Joris et al., 1998). After this step, the micropipette is washed with fluoric acid solution for a few seconds. The purpose of this washing is to remove glass dust and particles remaining in the pipette (Ward and Yanagimachi,

2018). After this step, the re-washed pipettes are placed in the Microforge and rotated up and down at 1500 RPM to allow the liquid in the pipette to come out. The pipette, which gets rid of the liquid inside, is placed horizontally and exposed to dry sterilization at 120°C (Joris et al., 1998).

The construction of holder pipettes is less laborious than the manufacture of manipulator pipettes. The pipettes placed in the horizontal directional pipette puller are pulled until they reach the desired fineness. The most suitable fineness level for a manipulator pipette is 15-20 μm inside diameter, while its outer surface is 60-80 μm in diameter (Van Steirteghem et al., 1993, Van Steirteghem et al., 1996). The pulled pipettes are broken with the help of micro sharpener and the broken pieces are formed into a round line by the sharpener. After this stage, the roughness at the tip of the holder pipette is removed with the help of fire in the microoven and it takes on a smooth structure. What is done after this step is the same as the steps of the manipulator pipette. Prepared and sterilized pipettes are placed in plastic containers or ready-to-use pipette holders and removed for use.

The shaping of the straws is adjusted to suit the task they are to perform. Both the inner and outer diameter of the holding pipette are much thicker than the manipulator pipette, and the tip is blunt. The manipulator pipette, on the other hand, is very thin and sharp compared to the holder pipette. The reason for this is to facilitate the insertion of the manipulator pipette into the oocyte and to cause less damage to the oocyte. The above mentioned pipettes are commercially sold by many companies. These straws, which are specially produced according to their duties, are illustrated in **figure 13** and **figure 14**. In order not to cause degeneration in the oocyte and to facilitate manipulation, an apparatus called a piezo pipette is used. Thanks to this apparatus, the manipulator pipette emits very small vibrations and transmits this vibration to the tip of the pipette. These extremely small vibration waves are called piezo waves. Thanks to these waves, the insertion of the pipette into the oocyte becomes easier.

Figure 13: Commercial micropipette

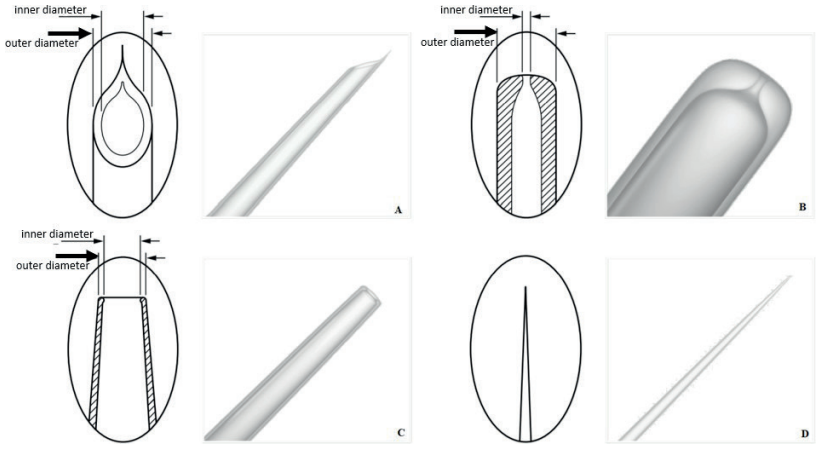

Figure 14: The pipette indicated by the letter A is a microinjection pipette. The tip of this pipette is oval and the tip is pointed to providea comfortable entrance to the oocyte. The pipette holder indicated by theletter B is the pipette. In this pipette, the outer diameter is much thicker thanthe inner diameter and is used to hold the oocyte during microinjection. It isused in the above-described "puncture of part of the zona pellucida" methodwith the pipette indicated by the letter C. Thanks to its oval tip, it helps to puncture the shingles layer around the oocyte in an oval shape. Finally, the pipette indicated with the letter D is used in the method of "cutting apart of the zona pellucida", which is an auxiliary technique, which is also mentioned above. Therefore, the tip ends in a pointed way to facilitate the cutting process.

5.3. Preparation of Sperm

The sperm retrieval process is applied differently in humans and animals. Different types of semen collection methods are used in different animal species. For example, in mice, the animal to be semen is killed and the epididymis is removed. An incision is made in the distal (thicker side) part of the epididymis held by the proximal (thinner side) and flowing semen is poured into a 1.5 mL microcentrifuge tube. 0.5 mL of CZB-HEPES medium is added to it and kept at 37°C for 10 minutes. Motile spermatozoa accumulated in the upper parts of the solution are collected. T6 medium is used in humans for this procedure. Sperm mixed with 0.5 mL of T6 medium **(Table 3)** in a 1.5 mL microcentrifuge tube is centrifuged at 1800 rpm for 5 minutes. At the end of centrifugation, the upper supernatant is removed and the remainder is resuspended with 20-50 μL of T6 medium. 2-4 μL of semen taken from the suspension is transferred to 1 g/10 ml of polyvinylpyrrolidone (PVP) solution to be used (Van Steirteghem et al., 1993).

Table 3: T6 medium

MEDIA CONTENT	T6 (mM)
NaCl	99.4 mM
KCl	1.42 mM
MgS04·7H20	0.71 mM
Na2HP04	0.36 mM
CaCI2·2H20	1.78 mM
NaHC03	25 mM
Glucose	5.56 mM
Na pyruvate	0.47 mM
Na lactate	24.9 mM
Penicillin	100 IU/ml
Streptomycin S04	50 μg/ml
Phenol red	0.001 %

In order to achieve success in ICSI applications, the quality of spermatozoa is as important as the quality of the oocyte. At this stage, the difference between the high quality and poor quality spermatozoa is revealed by several different separation methods. Separation of spermatozoa from bacteria, dead cells and various particles is ensured.

Below are some of these methods.

5.3.1. Swim Up Method

The swim up method, which means up-floating in Turkish, is based on the principle of separating motile sperm (the smooth, linear, active and stable movement of spermatozoa in the direction of the head) by floating them from the others. For this method, semen frozen in pellets or liquid straws is used. The semen, which are thawed for 25 seconds at 38.5°C, are poured into 15 ml cone-shaped tubes at a dose of 1 ml. Equal volume of Tyrode's Albumin Lactate Pyruvate (TALP, 114 mM NaCl, 3.1 mM KCl, 0.3 mM NaH2PO4, 2.1 mM CaCl2, 0.4 mM MgCl2, 2 mM NaHCO3, 0.2 mM sodium pyruvate, 10 mM sodium pyruvate, 10 mM sodium pyruvate) ml gentamicin sulfate, 10 mM Hepes, and 3 mg/ml BSA) medium or similar suitable media can be added. These media are used in both the swim up method and the spermatozoon washing method. The 15 ml conical tube in which the medium-sperm mixture is placed is kept at an angle of 45° in an incubator containing 5% CO_2 at 38.5°C for 30 to 60 minutes. During this time, motile spermatozoa swim to the upper part of the medium, while non-motile spermatozoa, various cells and particles remain at the bottom of the medium. The spermatozoa remaining on the upper surface of the medium are collected with the help of a micropipette and fertilization drops are prepared. The advantage of this method is that at least 90% of the semen whose density is determined by the hemocytometric method are motile. Obtained spermatozoa are free from contamination factors (Centola, 2005; Acar, 2011).

5.3.2. Sperm Washing Method

In the ordinary semen washing system, all living and dead cells are obtained. The thawed semen as described in the swim up method is placed in the centrifuge tube and a washing solution is added to it, 2 or 3 times the amount of semen. The same volume of water-filled centrifuge tube is placed in front of the semen that reaches its final volume with the added washing solution and centrifuged at 280-300 RPM for 10 minutes. At the end of the centrifugation, semen and other cells accumulate at the bottom, while the supernatant liquid remains at the top **(Figure 15)**. With the help of a pipette, the supernatant is removed and replaced with a new one, and the semen and medium are mixed again. After mixing, centrifugation is performed once again in the same way and the supernatant is removed. The last remaining portion is resuspended in culture medium and made ready for injection.

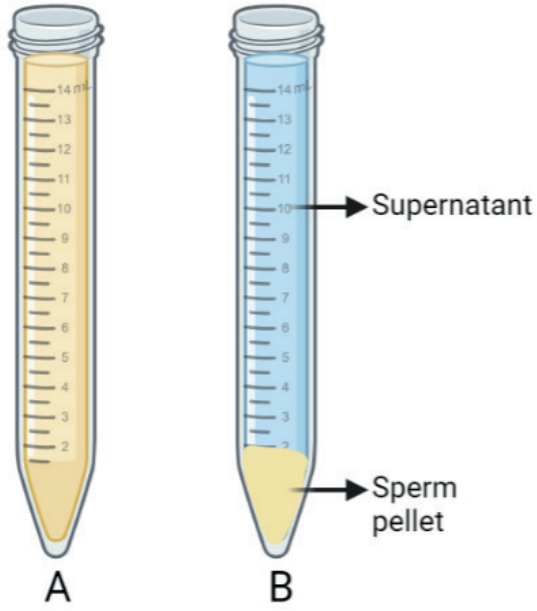

Figure 15: Sperm preparation for insemination.

This system is the most common system in terms of easy construction. It also allows the removal of cryoprotectants containing egg yolk, glycerol and chemicals in the extender (Acar, 2011). Although the system in question is advantageous in terms of easy implementation, it also has many disadvantages. At the end of the centrifugation process, there are dead cells, white blood cells (WBC), various microorganisms and many particles, as well as high quality spermatozoa at the bottom. Since it is known that white blood cells and dead cells also form reactive oxygen groups (ROS), the presence of ROS, which has a very harmful effect on the membrane of the spermatozoa and organelle membranes, reduces success (Mortimer, 2000; Centola, 2005).

5.3.3. Percoll Density Gradient

Percoll is defined as a colloidal suspension of silica particles coated with polyvinylpyrrolidone (PVP) (Acar, 2011). While this method was widely used in the 1980s, its use to separate human semen is now banned due to its high endotoxin content. Likewise, its use as a technique for separating animal semen has decreased. The Percoll density gradient method can be explained as follows. 90% percoll, 45% percoll and semen are placed in a centrifuge tube from bottom to top, respectively. The tube is centrifuged

at 700 RPM (Parrish et al., 1995) for 30-45 minutes. At the end of the centrifugation process, it is seen that motile spermatozoa are located on the lowest surface of the tube. Wolf et al (2008) achieved pregnancy by using percoll and swim-up methods in their study on cattle, and as a result, they compared the genders. As a result of this study, which is based on the information that male spermatozoa are lighter than females, they go faster than females, it was determined that more male offspring were born in the pups obtained by the swim up method compared to the pups obtained by the percoll method.

The Percoll method is more advantageous than the other two methods in terms of obtaining the highest motility rate, eliminating reactive oxygen groups and separating them from leukocytes. Despite these, it is not a preferred method today as the procedures are expensive, contain a high level of endotoxin and are not used much in the field of ICSI.

5.3.4. Immobilization of Spermatozoa

The spermatozoa, whose quality is determined by the methods mentioned above, are transferred to the PVP drops for the immobilization process and mixed. 10 spermatozoa, which are thought to be beautiful, are taken from the transferred spermatozoa and transferred to another PVP drop. The spermatozoa transferred to the second PVP drone are first pulled into the pipette starting from their tails and the head is left out so that their necks are at the tip of the pipette. The head and tail are separated from each other by pressing the neck with the tip of the micropipette. The same procedure is applied to all spermatozoa and at the end of the procedure, the head of the spermatozoa is pulled to the manipulator micropipette and made ready for injection **(Figure 16)**.

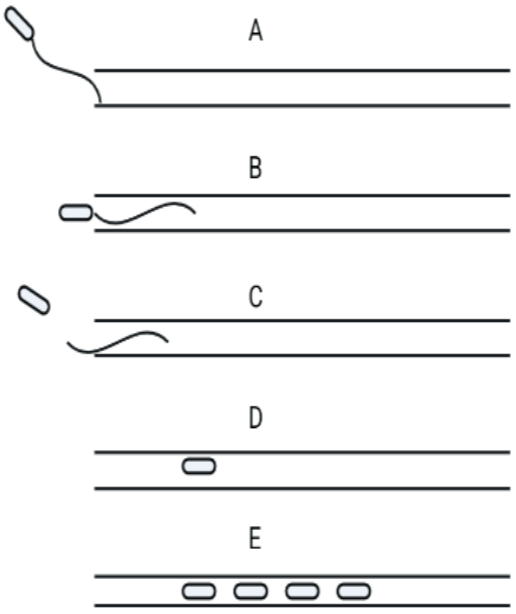

Figure 16: In this figure, the drawing of the semen into the pipette in the part indicated by A, its positioning in the picture B, the separation ofthe head and tail in the picture C, the aspiration of the head in the pictureD and the positioning of the heads of the spermatozoa in order to make consecutive injections in the picture E are shown.

During this process, the question arises why the head and tail of the spermatozoa are separated. In response to this question, Yanaihara et al. (2005) point out 2 factors as the reason. The first of these is the need to restrict the movement of spermatozoa. In order to facilitate the injection, the spermatozoa are drawn into the micropipette, first their tails and then their heads. In this way, the head of the spermatozoon is forward during the injection and it can be released into the ooplasm more easily. However, after motile spermatozoa are drawn into the pipette, they continue to move and move and all spermatozoa can be collected at the entrance of the pipette in multiple injections. In this case, manipulation and injection becomes very difficult.

While the spermatozoa are drawn to the micropipette, some PVP medium is also drawn with the pipette. Since these extracted mediums are transferred into the oocyte during injection, they are aspirated back to eliminate their harmful effects. This process makes micromanipulations made at such a small level difficult, damages the oocyte and prolongs the healing process. The

second factor is aimed at facilitating fertilization. According to Yanaihara et al. (2005), the pronucleus in sperm is formed as a result of decondensation of the nucleus, which is formed by damage to the membrane of the sperm. On this subject, Palermo et al. (1996) used 2 different (standard immobilization, aggressive immobilization) immobilization methods in epididymal semen, frozen-thawed epididymal semen, testicular semen and fresh semen in their study in humans and it was determined that the aggressive immobilization method gave better fertilization results in all semen. This is illustrated in more detail in **Figure 17**.

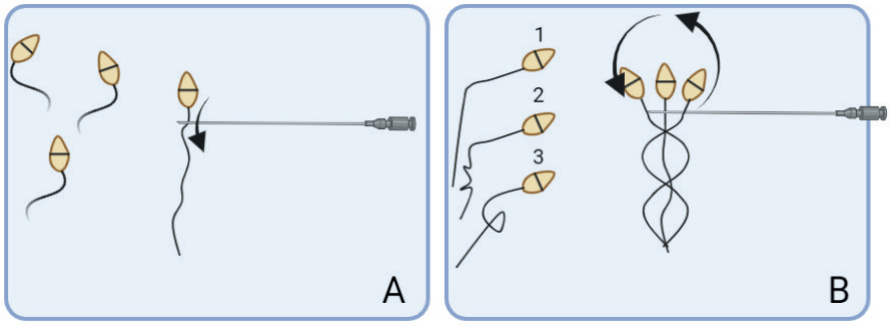

Figure 17: The standard immobilization pattern is shown as A, and the aggressive immobilization pattern is shown as B. According to the study, although aggressive immobilization gives better results, problems may occur in spermatozoa from time to time. While the format shown in number 1 is the desired format, undesirable situations shown in numbers 2 and 3 may also occur.

In cattle, fresh or frozen-thawed semen is used. The collected semen is placed in a centrifuge tube and centrifuged in medium containing 20mM HEPES (10 minutes at 300 RPM). 0.1mL of washed semen is taken and transferred into 5mM dithiothreitol (DTT) and left for 1 hour. DTT causes the weakening of the proteamine disulfide bonds at the head of the spermatozoa and decondensation in the nuclear chromatin of the head of the spermatozoon, resulting in a pronucleus (Suttner et al., 2000; Galli et al., 2003). To separate it from DTT, it is centrifuged 2 times in HEPES at 350 RPM for 10 minutes and transferred to the micromanipulator drone. In order to slow down the motile spermatozoa, 3μL of semen is transferred into 8% PVP medium. 2 drops of 5 μL each are prepared in 6 cm petri dishes. One contains spermatozoa in PVP and the other contains 3 or 4 oocytes. After the spermatozoa are immobilized in the above-mentioned manner, injection is made (Li et al., 2003).

5.4. Oocyte Injection of Spermatozoa

In this section, the process of intracytoplasmic sperm injection, which is the main procedure, will be discussed after the appropriately retrieval and maturation of oocytes according to the selected animal species, and the preparation of semen during maturation.

Petri dishes are prepared for ICSI application. First, a line is drawn on the back of the petri dish to be treated, and the petri dish is divided into 2 parts, up and down **(Figure 18a)**, then 5μL PVP drops are prepared on the upper part of the drawn line **(Figure 18b)**. The drops above the line are reserved for semen-related operations. After this step, 20 μL drops are prepared at the bottom of the line. Although the content of these drops differs according to the solution used, HEPES/CZB or Whitten's/PVA drops with similar properties are usually prepared **(Figure 18c)**.

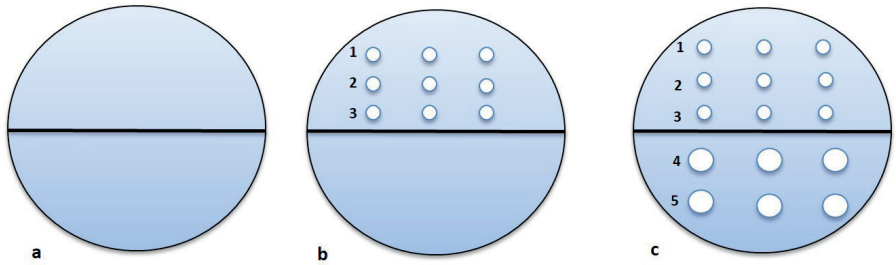

Figure 18: Preparation of the ICSI application petri dish

Prepared drops are classified as 1, 2 and 3 from top to bottom. The first drop series (number 3) above the line is used as semen storage. Selected motile spermatozoa are placed in these drops and spermatozoa to be selected are selected from these drops. The 10 selected spermatozoa are removed to a top layer, that is, to the number 2 drops. Here, the spermatozoa are washed, their heads are separated from the tails and immobilized, and the oocyte is kept ready for injection. A top layer, number 1, is used to clean various residues that may be found in the pipette after manipulation. Of the 2 layer drops at the bottom of the line, the ones in the first layer (number 4) are used as oocyte storage and ICSI applications are made in these drops. The drops in the lowest layer (number 5) are used as adjustment drops for the correct positioning of the micropipettes. This system can be rearranged according to the user's ability to manipulate easily and the mobility of the manipulator. A few examples of this situation are shown in **Figure 19** (Yanaihara et al., 2005).

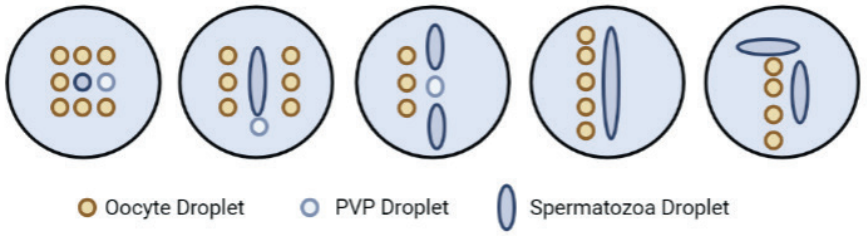

Figure 19: Alternative ICSI application plates

ICSI applications are often made in 60 mm plastic petri dishes. The first polar body (PB) is generally positioned at 6 and/or 12 o'clock. The zona pellucida is pierced with the help of piezo waves and the injection pipette enters the oocyte without damaging the zona pellucida. Before the tip of the pipette reaches the middle part of the oocyte, the oolemma is passed and the semen is left in front of the trapping pipette by advancing to the holding pipette. The important point here is that the spindle fibers in Metaphase II are located under the 1st polar body (Hardarson et al., 2000). Stoddart et al., in their study, observed that positioning the polar body at 12 o'clock during ICSI achieved higher success than positioning it at 6 o'clock (Stoddart and Fleming, 2000). Blake et al. emphasized that the best quality embryos were obtained from oocytes with PB positioned at 11 o'clock and 7 o'clock in their study (Blake et al., 2000). In their study, Yanaihara et al. (2005) divided the oocyte into 9 equal parts, in which they arranged the polar body to be positioned at 12 o'clock, and injected semen into different parts by piercing the zona pellucida with an injection pipette at 3 o'clock. The study and its results are shown in **Figure 20**.

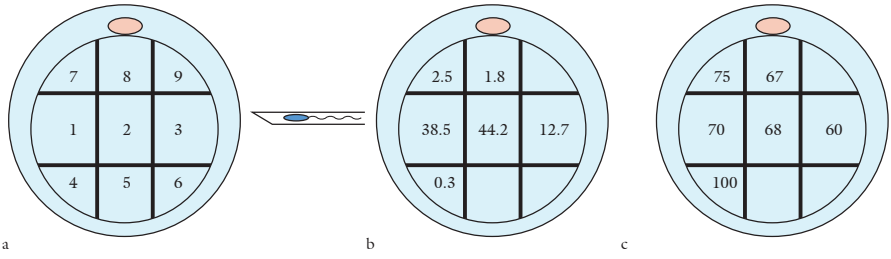

Figure 20: In the picture shown with the letter a in Figure 18, the ooplasm is divided into 9 parts and numbered. In the part shown with the letter B, the part where the semen is left and the positioning situation are shown. In the study, it was observed that 80% of the spermatozoa were located to the left of the midline. In the part indicated with the letter C, the fertilization rates according to the regions were specified and the lowest rate of fertilization was observed in the semen left in the 3rd part to the right of the midline (Yanaihara et al., 2005).

It is beneficial to aspirate the PVP medium that is involuntarily injected into the ooplasm during the sperm release or the semen buffer medium used. Ward et al. (2018) schematized the intracytoplasmic sperm injection as in **figure 21**. This shape was made by Ward and Yanagimachi to schematize their ICSI applications.

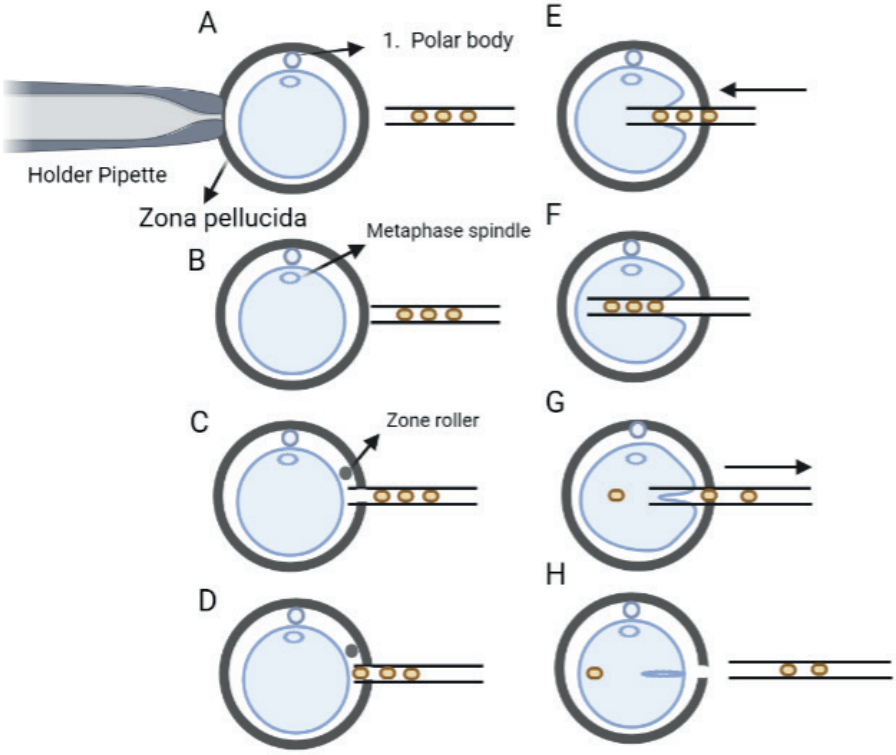

Figure 21: The ICSI process is photographed in Figure 21, the passage of the zona pellucida in the figure shown with C, the entrance to the oolemma and reaching the injection site in the figure shown with E, the release of the semen in the figure shown with F and G, the back aspiration of the semen injection fluid and the position of the semen and their exit from the oocyte are in the figure shown with H (Ward and Yanagimachi, 2018)

After the injection process is finished, the survivors are selected from the samples that are kept at room temperature for 1 hour and transferred to culture drops prepared based on CZB, Synthetic Oviduct Fluid (SOF) or Charles Rosenkrans (CR), and their development is observed at 38.5 °C in an environment containing 5% CO_2. Kimura and Yanagimachi investigated the healing process of oolemma after puncture and emphasized that healing

is better at lower temperatures. They depicted this situation as in **figure 22** (Kimura and Yanagimachi, 1995).

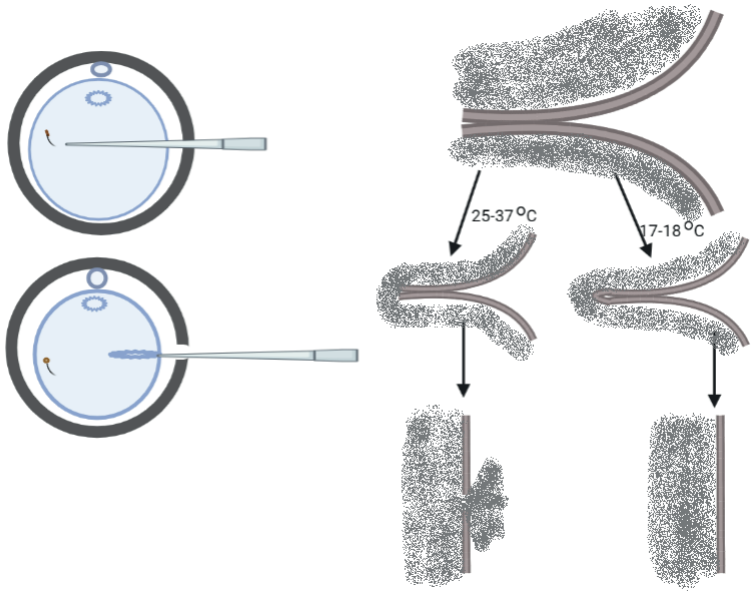

Figure 22: The puncture of the oolemma during injection and the damage of the manipulator pipette to the oolemma are shown. Under normal conditions, the oolemma shortens and returns to its original state and the wound lips are closed, but at temperatures of 25-37 °C, the recovery process accelerates and the plasma membrane takes its original form before it can heal. In this case, ooplasm exits and may cause the oocyte to fail to develop. When the ambient temperature is lowered, the recovery time of the oolemma increases and the wound lips have enough time to unite. In this case, the oocyte is not damaged and can continue to develop.

5.5. Oocyte Activation

Oocyte activation is a series of events that transforms the metaphase II oocyte into a fertilized egg ready to initiate embryogenesis. Calcium waves created by the spermatozoon cause stimulation of the cortical granules when the sperm enters the oocyte and comes into contact with the ooplasm. The cortical granules separated from the ooplasm gather under the zona pellucida and combine with the zona pellucida, changing the structure of the zona pellucida. In this way, the oocyte combined with the sperm forms the polyspermy block, which protects the oocyte against external factors and prevents other sperm from attaching to the zona pellucida and passing through the zona pellucida. The oocyte cell completes metaphase II to complete the cell cycle and allow the formation of the female pronucleus

that fuses with the pronucleus from the sperm. With the formation of the cytoskeleton, the zygote is protected and embryo formation takes place.

The most important key that initiates all these changes is the amount of calcium that increases with the spermatozoon that enters the oocyte to perform fertilization (Ramadan et al., 2012). The increase in intracellular calcium mostly occurs due to the release of calcium in the endoplasmic reticulum (Wakai and Fissore, 2013). While the increase in calcium in in vitro fertilization starts with the penetration of the sperm into the oocyte, it is shaped by the influx of calcium from the ambient fluid during the provoked injection such as intracytoplasmic sperm injection (Tesarik et al., 2000).

With the increase in the amount of calcium in the cell, calcium-sensitive cellular receptors are stimulated and oocyte activation is initiated. Scientists stated that spermatozoa secrete phospholipase-C-zeta (PLCζ) protein after entering the oocyte, and this protein causes calcium release from the endoplasmic reticulum of the oocyte via inositol-1.4.5-triphosphate into the oocyte cytosol (Saunders et al., 2002; Aarabi et al., 2012). There are three types of artificial activation methods for oocyte activation: mechanical, electrical and chemical activation.

1. **Mechanical Activation:** It is based on the puncture of the zona pellucida to ensure calcium entry into the oocyte. Since the zona pellucida is damaged during these procedures, there is a risk of contamination and deterioration of the integrity of the oocyte.
2. **Chemical Activation:** It is the activation method made by adding some chemical substances that increase the calcium in the oocyte to the culture medium after microinjection.
3. **Electrical Activation:** During microinjection, low-voltage electrical waves are given to the oocyte, causing the calcium in the oocyte endoplasmic reticulum to come out. At the same time, the given electricity also changes the structure of the phospholipids in the cell membrane, causing the calcium channels in the cellular pores to open, and it is a method based on the activation of the calcium entering the cell. During this procedure, Zimmerman mammalian cell fusion medium **(Table 4)** (Bertsche et al., 1988) and Zimmerman cell fusion apparatus **(Figure 24)** are used (Ware et al., 1989). There are copper electrical probes on the right and left parts of the fusion apparatus. One probe is positively (+) and the other is negatively (-) charged **(Figure 25)**. In order to fix the probes, a partition apparatus is placed on the right and left, and glass partitions are placed above and below.

In this process, the oocytes taken from the culture medium are washed 2-3 times with Zimmerman mammalian cell fusion medium and the oocytes are expected to settle to the bottom. After the oocytes sink to the bottom, the fusion medium is left between the copper wires in the fusion apparatus and 110-120 V electricity is applied for 30 μs. In some studies, 4V electricity is also applied for 10 seconds (Wang et al., 1998).

Table 4: Zimmerman Mammalian Cell Fusion Medium

MEDIA CONTENT	**Zimmerman Mammalian Cell Fusion Medium (mM)**
Glucose	27 mM
NaCl	70 mM
KCl	3.4 mM
KH2PO4	0.6 mM
Na2HPO4	1.1 mM
Calcium acetate	0.1 mM
Magnesium acetate	0.5 mM

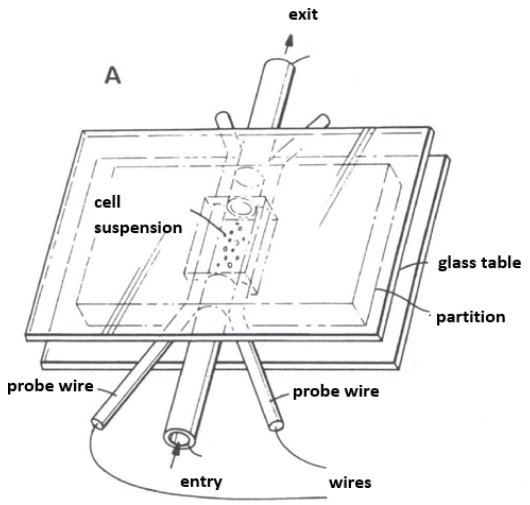

Figure 23: The Zimmerman cell fusion apparatus is fixed with 2 glass plates from the top and bottom, and is separated from each other by a partition for placing the probes and ensuring cell flow between the probes. The probes are located on the right and left in the middle of the intermediate chamber. The probe cables, which provide the transmission of electricity, enter from the bottom of the apparatus and exit from the upper part. There is a tube between the probe cables. From this tube, the cell suspension is left between the probes and after the electrification process is finished, it is taken out of the apparatus with the same tube system as the cell.

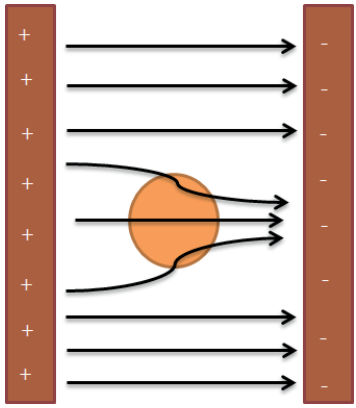

Figure 24: The cell passing between the probes is placed between the positively charged probe and the negatively charged probe and is expected to sink to the bottom. Electricity is applied to the sinking oocyte and its activation is initiated.

After the electrification process is completed, the oocytes are collected again and washed with the culture medium and then left to incubate in the new culture medium at 38.5°C in an environment with 5% CO2.

Many scientists have used various methods for oocyte activation. Some of these are listed below.

- Ionomycin (Ware et al., 1989).
- Ethanol (Nagai, 1987).
- 6-dimethylaminopurine (DMAP) (Keskintepe et al., 2002).
- Electrical Stimulation (Kono et al., 1989).
- Cycloheximide (Sirard et al., 1989).
- Cytosialysin (Lagutina et al., 2004).
- Puromycin (Murase et al., 2004).

Chung et al. (2000) investigated the effects of different substances on oocyte activation in their study in cattle, used combinations of 5μM ionomycin, 5μM ionomycin + 1.9mM DMAP and 5μM-50μM ionomycin and found that the most effective method was 5μM ionomycin + 1.9mM DMAP. While this situation has been demonstrated in cattle in this way, Wang et al. (1998) emphasized that electrical stimulation is more effective than chemical activation of the pig oocyte in their study in pigs. Collas et al. (1993) investigated the effects of electrical and chemical stimulation in their study on bovine oocytes.

While electrical stimuli affect the Ca^{+2} increase very rapidly, ionomycin administration provides small increases in the temporary Ca^{+2} increase. As it can be understood from here, different methods of stimulating activation are used in different animal species. While stimulating oocyte activation by chemical method is more effective in cattle, electrical activation is more effective in pigs.

6. Short- and Long-term Risks of ART Technology

The number of couples undergoing IVF treatment continues to increase rapidly day by day. Worldwide, 5 million babies have been born thanks to ART, and today, in vitro fertilization accounts for approximately 2% of all deliveries. Therefore, information on the long-term health of children born by in vitro fertilization is a matter of great interest. Babies born with ART have a higher danger of birth defects. Many studies have suggested an advanced risk of birth defects in babies born with ART compared to babies born naturally (Bonduelle et al., 2005). The miscarriage rate in pregnancies obtained by in vitro fertilization was reported to be 24-30% higher than in natural pregnancies. It has been reported that aneuploidy is most common in X, 22, 21, 13 and 4 chromosomes and is found in roughly half of the embryos that spontaneously abort (Tan et al., 2004).

It was observed that defects occurred in various systems of the body, but when evaluated in terms of prevalence, it was observed that there were no significant differences. It has been suggested that the effect on the nervous system is higher when compared to the face, eyes, ears and neck. Moreover, it has been suggested that the probability of having more than one birth defect is high in children born with ART, and that there is a relationship between cerebral palsy, a neurological disorder and ART (Hansen et al., 2002; Stromberg et al., 2002). Recent studies have also revealed that ART disrupts glucose metabolism, increases fasting glucose levels and causes insulin resistance (Tamashiro et al., 2002; Ceelen et al., 2008; Scott et al., 2010). In an animal study, it was suggested that ART-treated mice had a hyperinsulinemic response to the intraperitoneal glucose tolerance test, although they had similar body weights to control mice (Scott et al., 2010). In line with these results, it can be said that mice born with ART are insulin resistant independent of obesity. According to a different study, ART might raise the risk of cardiovascular disease (Watkins et al., 2007). Watkins et al. found that mouse pups raised in vitro from the two-cell to early blastocyst stage had higher systolic blood pressure at 21 weeks of age (Watkins et al., 2007). Another study in humans reported that systolic and diastolic blood pressure levels were higher in children born with ART than in naturally

born children (Ceelen et al., 2008). In addition, another research article on animals suggested that ART may affect thyroid function. It has been reported that serum T3, T4 and potassium concentrations of calves obtained from in vitro fertilized embryos are lower than those obtained from artificial insemination (Rerat et al., 2005). In another human study, it was reported that higher thyroid stimulating hormone (TSH) levels were observed in IVF children compared to naturally born children (Sakka et al., 2009).

Despite the abundance of evidence indicating that ART heightens the likelihood of both immediate and enduring complications, the underlying factors responsible for this outcome remain a topic of ongoing debate and lack a definitive consensus. The causality of complications arising from ART treatment remains uncertain, as it is unclear whether the adverse outcomes are attributable to the ART procedure itself or to other factors associated with the infertile couple receiving ART therapy. Genomic instability may be caused by the ART procedure or the infertile history of couples, as reported in literature (Feng et al., 2008). According to a particular study, there was a marginal escalation in the instability of dynamic mutations in the offspring who were conceived through assisted reproductive technology (ART) in comparison to the offspring who were conceived naturally, despite the fathers having normal spermatogenesis and genetic backgrounds. The presence of instability could potentially indicate an issue with infertility, as well as controlled ovarian hyperstimulation and/or conditions related to in vitro culture.

In the ICSI method, a single sperm cell is injected into the oocyte through micromanipulators. By using this method, even sperm with impaired motility and sperm with abnormal morphology have the ability to fertilize the oocyte. As a result, there are worries about the safety of ICSI and the genetic risks associated with children born using this procedure. Because the selection of a morphologically normal sperm under the microscope while using the ICSI method might not be as precise as in vivo. Babies born through this procedure may be more likely to have problems if sperm with poor motility and morphology is used. When infertile men diagnosed with oligozoospermia or azoospermia have children with the ICSI method, the incidence of chromosomal abnormalities such as Y chromosome deletions and abnormal karyotypes was found to be high in the offspring of these men (Simpson and Lamb, 2001). In addition, since the natural selection in the oocyte membrane, which occurs in both natural pregnancy and classical IVF, does not occur in the ICSI method, it allows genetically and structurally abnormal sperm to fertilize the oocytes and to carry the abnormal genetic material to the offspring (Lu et al., 2013). Moreover, unlike classical IVF, it

is possible for foreign materials to enter the oocyte following ICSI since the cell membrane of the oocyte is physically disrupted (Bowen et al., 1998).

Mutations can also occur in the ICSI method as a result of in vitro chemical and environmental exposures (Simpson and Lamb, 2001). According to the results reported from prenatal diagnoses, it has been reported that pregnancies obtained by the ICSI method tend to have a higher frequency of aneuploidy compared to naturally conceived children (Tournaye et al., 1995; In't Velt et al., 1995; Bonduelle et al., 2002; Van Steirteghem et al., 2002). The genetic consequences of the use of the ICSI technique in babies born with the ICSI method have been investigated by many researchers (Bonduelle et al., 2002; Bonduelle et al., 1999). It has been reported that de novo chromosomal abnormalities are associated with 1.66% of autosomes and sex chromosomes, and 0.92% of hereditary structural chromosomal abnormalities inherited from the father. As a result of the evaluations, it has been shown that the main congenital abnormalities are equal to 2.3% of the total number of children born. When fetal deaths were evaluated, it was observed that it was 1.1% after the 20th week of pregnancy. Another study compared data between ICSI (n =889) and IVF infants (n =995) born between 1991–1999 and 1983–1999. It has been reported that the ICSI group did not show an increased risk of major malformations or complications compared with the IVF group (Bonduelle et al., 2002). It has been reported that children born by the ICSI method show a higher incidence of hypospadias when compared to children born naturally. However, no other situation that could pose an extreme risk was observed, suggesting that this may be related to the hormones taken by the mother at the beginning of her pregnancy or the father's subfertility (Wennerholm et al., 2000; Ericson and Kallen, 2001). Although genetic factors are known to be effective in the etiology of azoospermia (Peschka et al., 1999; Vegetti et al., 2000), it has been argued that infants born from epididymal or testicular spermatozoa by ICSI method do not have higher risks for congenital abnormalities compared to naturally-born infants (Bonduelle et al., 1998; Bonduelle et al., 1999; Bonduelle et al., 2002). In addition, a high incidence of congenital abnormalities was not observed in children born from pregnancies obtained by transferring frozen-thawed embryos obtained by the ICSI method.

In another study evaluating data on 139 children born by the ICSI method, congenital abnormalities were observed at the level of 7.6% and half of these abnormalities were at the minor level (Wennerholm et al., 2000). In this study, while the relative risk of congenital abnormality was 1.75% in children born with ICSI, this risk decreased to 1.19% in twins or

triplets. The only congenital abnormality observed at an alarmingly high rate was hypospadias with a rate of 3%. In another study in which post-ICSI techniques were applied, it was suggested that the somatic development of children was normal (Van Steirteghem et al., 2002). To assess the mental development and fertility of children born with the ICSI technique, long-term research are required. It may be advised to do cytogenetic analysis of haploid male gametes from ejaculates or testicular biopsy samples before ICSI method is used in men with low sperm count or azoospermic in order to decrease the potential dangers of ICSI method in foetuses or neonates. Two distinct investigations, one after the other, looked at the mental and neuropsychiatric growth of kids born using the ICSI technique. With the exception of issues related to multiple pregnancies (Bonduelle et al., 1998; Van Steirteghem et al., 2002), no conclusions have been drawn to support a major abnormality in children born by the ICSI method or a significant departure from the normally naturally conceived population based on findings regarding the presence of hypospadias (Wennerholm et al., 2000; Ericson and Kallen, 2001). According to a different study (Bonduelle et al., 2005), infants born through the IVF and ICSI methods required more medical resources than children born spontaneously. Additionally, it was found in this study that children born using the ICSI technique have a higher rate of congenital abnormalities.

Polyvinylpyrrolidone (PVP) is employed in the Intracytoplasmic Sperm Injection (ICSI) technique to enhance the sperm cell's viscosity and promote the transportation and mobility of the sperm. As a result of the studies, it has been reported that PVP causes significant damage to the membrane of the sperm cell. It has even been found that PVP solution is found in embryos to be transferred to the mother, and pregnancies with the embryos created by ICSI method are associated with chromosomal abnormalities (Jean et al., 2001; Parmegiani et al., 2010).

7. Epigenetic Risks of ICSI

Interest in the epigenetic effect of the ICSI method has increased in recent years. Epigenetics emerged as a discipline based on the concept of 'epigenesis'. Epigenesis provides a framework for how various cell phenotypes can evolve from a single set of identical genetic code. Epigenetics is the study of inherited changes in gene expression that occur without a change in the DNA sequence, by transferring them to the next generation. Epigenetic modifications include DNA methylation, histone modification, remodeling of nucleosomes, higher order chromatin rearrangement, and regulation by noncoding RNAs. Epigenetic modifications can affect patterns

of gene expression required in embryonic, fetal and postnatal development (Le Bouc et al., 2010). DNA methylation is the most studied epigenetic mechanism. Changes in DNA methylation and the frequency of imprinting disorders have been associated with ART (Manipalviratn et al., 2009). Gametogenesis and early preimplantation development are characterised by two distinct waves of DNA methylation and demethylation (Reik and Walter, 2001). The procedures of ovarian stimulation, assisted fertilisation, and other in vitro manipulations utilised in IVF have the potential to hinder the establishment of DNA methylation in gametes. This is due to the maintenance of DNA methylation in preimplantation embryos (Falls et al., 1999). DNA methylation is the most studied epigenetic process related to fertility treatment and imprinting syndromes. An important event in early development is imprinting, in which genes are epigenetically regulated and expressed according to parental origin. The disruption of genomic imprinting has been identified as a causative factor in the manifestation of various genetic syndromes, including but not limited to Beckwith-Wiedemann syndrome (BWS), Prader-Willi syndrome, and Angelman syndrome (AS) (Feng et al., 2011).

Potential epigenetic impairments, including syndromes such as Angelman syndrome (AS), Beckwith–Wiedemann syndrome (BWS), Silver-Russell syndrome (SRS), have been reported in babies born by ICSI in many studies. Asperger syndrome (AS) is a neurogenetic condition characterised by profound mental impairment, delayed motor and balance development, speech impediment, or surprisingly cheerful behaviour. Due to genetic changes that decrease the activity or expression of the UBE3A gene on chromosome 15, this condition, which affects 1 in 20,000 children, is present (Cox et al., 2002; Elgersma, 2015). Case studies have led to the hypothesis that ICSI for infertile couples may raise the likelihood of an imprinting defect leading to AS (Cox et al., 2002; Orstavik et al., 2003). According to a cohort research, couples who underwent hormonal stimulation or ICSI had a considerably greater chance of having a kid with an imprinting issue that causes AS (Ludwig et al., 2005). There are studies in the literature that argue the opposite of this situation. In a research, it was reported that none of 92 children born via ICSI had clinical symptoms of AS (Manning et al., 2006). BWS is an extremely uncommon imprinting condition that affects roughly 1 in 13,700 live births. Macroglossia, midline abdominal wall abnormalities, macrosomia, newborn hypoglycemia, and several developmental malformations are the hallmarks of this congenital overgrowth syndrome condition. According to Manipalviratn et al. (2009), children with this disease have a very high chance of developing embryonal

tumours like Wilm's tumour, rhabdomyosarcoma, and hepatoblastoma. Chromosome 11p15 abnormalities are present in patients with this condition. Maternal and paternal genes (H19, IGF2) implicated in imprinting and the aetiology of BWS have been localised to the 11p15 region. DMR1 normally coordinates the regulation of the maternal H19 and the paternal IGF2 genes (Gosden et al., 2003; Chang et al., 2005; Manipalviratn et al., 2009). A distinct methylation pattern in CvDMR in embryonic and extra-embryonic tissues was proposed in a study where BWS cases were reported as a result of the ICSI procedure (Gomes et al., 2007). The ICSI approach was then used on a pair lacking the BWS phenotype's traits, and distinct maternal methylation patterns in KvDMR were found in DNA samples from various organs (Gomes et al., 2007). In another study, researchers found that loss of maternal allele methylation in differentially methylated regions occurred in 37.5% of ART-born BWS cases and 6.4% of non-ART-born BWS cases when they compared the clinical phenotype and molecular characteristics of 25 BWS children born to ART with those of 97 naturally-born BWS children. They discovered that ART may be linked to compromised normal genomic imprinting in children, which is consistent with the result they obtained (Lim et al., 2009).

These research have led to the conclusion that the ICSI technique considerably raises the likelihood of genetic abnormalities and imprinting issues. There are doubts about the validity of the ICSI technique because it skips some stages of fertilisation in a typical pregnancy and is invasive because the oocyte's cell membrane is physically damaged. To confirm whether this technique raises the risk of genetic abnormalities and imprinting disorders, larger longitudinal investigations are required. Additionally, it is yet unclear if these unfavourable circumstances are linked to the couples' genetic makeup or the therapy methods used. To determine the exact nature of the condition, thorough studies are required.

Conclusion

It is known that more than five million newborns (about 1-4% of the newborn population) have been born with ART worldwide. In assisted reproductive technology, fertilization is carried out by putting the oocytes taken in the laboratory into drops containing spermatozoa, since the fertilization that should occur in the ampulla isthmus region in the normal mating procedure does not occur due to various reasons for various reasons. A healthy oocyte and healthy spermatozoa are required for this method to be successful. IVF, which is a suitable alternative for fertilization that does not occur due to female-related reasons, does not work efficiently in male-related

problems. The ICSI method, which was developed as an alternative to this situation, eliminates the problems related to both women and men. The ICSI method is an alternative method to methods such as IVF and subzonal sperm injection (SUZI). This method has been used quite frequently in the world in terms of eliminating problems related to both men and women and has become the most common procedure applied over time. ICSI is an important strategy to increase ART live birth rates, especially in cases of male-factor infertility. Although ICSI is a useful treatment for infertile couples, it may increase the likelihood of adult disease in newborn babies. To limit the dangers of adult disease from gametes/embryos and to improve ART techniques, risk mechanisms should be further explored. More clinically controlled trials with standard techniques and larger sample sizes are needed. It is critical to learn better about the impact of epigenetics in infertility situations and the detrimental effects of fertility treatment on the health and fertility of future generations. The short- and long-term safety of the ICSI method should be carefully evaluated. Prospective epidemiological studies are needed to identify the specific hazards associated with ART operations and to comprehensively examine potential risk variables associated with adult disease.

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