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Laser Pulse Application in IVF

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1. Introduction

In-vitro fertilization (IVF) involves the culture and manipulation of gametes and embryos within a laboratory environment. IVF procedures are channeled towards enhancing fertilization and assisting the normal developmental physiology of the growing embryo to increase implantation potential, culminating in the birth of a healthy baby. Laser and its selective application to various steps in the IVF process is an area of growing interest.

In this chapter, we review the use of laser technology in the field of assisted reproduction as well as in stem cell research. The first step in the IVF process involves fertilization of the oocyte. For this to occur, sperm must penetrate the outer membrane known as the "zona pellucida" which surrounds the egg. This natural barrier prevents the entry of multiple sperm. Often it is necessary to assist fertilization by directly injecting a single sperm into the oocyte, a technique known as Intracytoplasmic Sperm Injection (ICSI). Laser pulse has been utilized to immobilize the human sperm tail before ICSI and in assisting the injection technique by creating a hole in the zona (laser assisted ICSI). Once successfully fertilized, the resulting embryo undergoes successive cell divisions. To implant on the uterine wall, the embryo must escape from the surrounding zona, a process known as hatching. Laser assisted hatching has been employed to create a controlled opening of the zona and facilitate embryo implantation after transfer to the patient's uterus. Zona opening through use of a laser pulse has also been used to extract a single cell from the growing embryo for preimplantation genetic diagnosis (PGD). Another application of the laser in reproductive biology has been cellular microsurgery. Embryonic stem cells can be isolated from a blastocyst stage embryo by selective ablation of trophectodermal cells, leaving behind the stem cell source material. More recently, laser has been used to induce fluid loss from the blastocyst stage embryo before cryopreservation. We discuss this novel application of laser and our own work with artificially collapsing blastocysts before freezing to reduce ice crystal damage.

This article also documents the evolution of laser pulse in IVF from the first generation of lasers with UV range wavelengths to the newer generation of lasers with emissions in the infrared range. Design characteristics for the ideal laser pulse for clinical IVF use are presented. Finally, safety considerations as regards laser usage at such early stages of development and potential risks to the newborn are discussed. The current FDA classification and approved devices are also reviewed.

Numerous engineering devices have been used in biomanipulation and a thorough understanding of both the disciplines of biology and engineering is imperative to develop

an efficient system for handling biological materials. Lab procedures used during IVF involve some of the newest innnovations in medical technology, which may be attributed to the constant pressure to increase accuracy and efficiency in completing procedures. Among these innovations is laser technology. With the replacement of mechanical manipulation by laser pulse, interuser variability may be lessened and consistently high laboratory standards may be maintained.

In vitro fertilization (IVF) is one of several treatment options used in assisted reproduction. It involves an interplay of diagnostic tests, hormonal supplementation, surgery and laboratory techniques to help the subfertile couple achieve a pregnancy resulting in a healthy baby. When a couple approaches the physician with the issue of subfertility, they undergo a series of tests to determine the cause of subfertility and the optimal assisted reproductive technique for their clinical situation. Causes of infertility may include lack of eggs (oocytes), lack of sperm, inability of egg and sperm to meet due to blocked fallopian tubes, inability to grow or implant in the uterus, or an unknown etiology.

In a typical IVF procedure, oocytes are harvested from the ovary after hormonal ovarian stimulation. A sperm sample is collected from the male partner and washed from surrounding semen. Alternatively, sperm is surgically retrieved from the testis or epididymis. The oocytes are allowed to naturally fertilize in a Petri dish by co-incubation with sperm. If the sperm count or motility is compromised, the insemination step is carried out by direct injection of each oocyte with a single sperm using a glass needle. This specialized procedure is known as ICSI (Intracytoplasmic Sperm Injection). If fertilization occurs, a zygote forms. The zygote divides, undergoing cell cleavage, and forms an embryo. The cells within the embryo continue rapidly dividing over the 4-6 day culture interval, ultimately arranging in a distinct pattern to become a blastocyst. The blastocyst consists of a peripheral layer of cells called the "trophectoderm" and a discrete grouping of cells known as the inner cell mass (ICM) that will eventually form the fetus (Figure 1). The developing embryo is protected by an outer shell of protein called the "zona pellucida" until it is large enough to break free during a process known as "hatching", in preparation for implantation into the uterine wall.

Couples will have multiple embryos developing simultaneously in culture. Each embryo is evaluated throughout its growth process. On the day of transfer 1-3 embryos are selected from the laboratory dish and transferred to the patient's uterus. This transfer may occur on day 3 or day 5 after fertilization. Any additional embryos that are appropriately developed are frozen for possible later transfer. Selection of embryos most likely to implant and lead to a viable pregnancy is generally based on embryo morphology.

While some applications of lasers in IVF remain research topics, others have been successfully employed in clinical practice. Laser assisted ICSI is used to aid fertilization. Laser assisted hatching has been employed to create a controlled opening of the zona and facilitate embryo implantation after transfer to the patient's uterus. Zona opening through use of a laser pulse has also been used to extract a single cell from the growing embryo for preimplantation genetic diagnosis (PGD) and screen for genetic disorders prior to transfer. Another application of the laser in reproductive biology has been cellular microsurgery. Embryonic stem cells can be isolated from a blastocyst stage embryo by selective ablation of trophectodermal cells, leaving behind the stem cell source material.

When first approaching the application of lasers to reproductive medicine, concerns were raised as regards the safety profile and class of lasers to be used. Given the delicate stage of human development at the time of fertilization, the major concerns regarding the use of

laser at earlier stages have been DNA damage, failed embryo development and possible congenital disorders. These concerns primarily centered on laser wavelength, heat generation and the amount of manipulation required of the fragile embryos. The primary aim of this review is to assimilate the significance and limitations of laser technology in the fast growing field of IVF and to outline the technical details to be considered when dealing with laser pulses in reproductive technology.

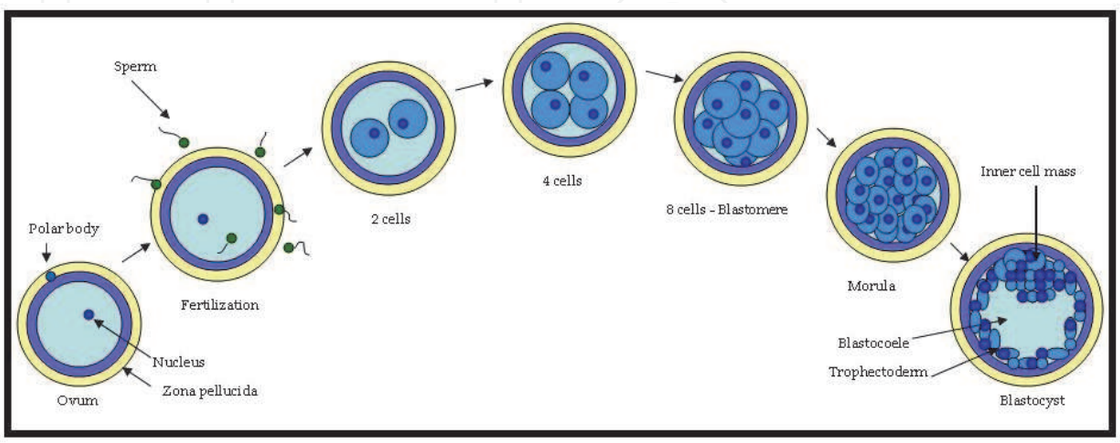

Fig. 1. Egg fertilization and development

2. History of lasers in IVF

Laser technology has been used in Assisted Reproductive Technology since the 1980s (Ebner *et al.*, 2005). Laser pulse has found wide application in IVF technology, particularly when efficient and precise manipulation is of paramount importance (Taylor *et al.*, 2010).

Two general types of laser systems exist: contact and noncontact. Noncontact lasers do not require additional physical manipulation of the embryo. Laser beams travel through the objective lenses and only microscope stage movement is required to adjust embryo position (Tadir *et al.*, 1989, 1990, 1991). In contrast, contact laser systems require direct contact between the laser and embryo, usually with either glass or an optical fiber (Neev *et al.*, 1992). This increases the likelihood of trauma to the embryo. Distance also affects damage – a greater distance from the embryo to the laser will result in a larger hole in the embryo, even if the difference in distance is only between the top and bottom of culture dish (Taylor *et al.*, 2010). Contact lasers also require use of a medium different than routine culture media in order to affect the most efficient energy transfer.

The first generation of lasers to be used in IVF included argon fluoride (ArF), Xenon chloride (XeCl), krypton fluoride (KrF), nitrogen and Nd:YAG lasers. The Nd:YAG laser (1064 nm) was the first non-contact laser used in reproductive technologies. Initial use was primarily for spermatozoa manipulation via optical trapping. Applications were then expanded to add a potassium-titanyl-phosphate crystal in order to create a hole in the zona pellucida to assist hatching (Tadir *et al.*,1989, 1990, 1991). Excimer lasers under development around the same time period function by temporarily exciting rare earth gasses. After comparing Nd:YAG lasers with the ArF (193 nm) excimer laser, the 193 nm was found to produce a more uniform, smooth tunnel in the zona pellucida (Palanker *et al.* 1991). Similar findings were noted with the XeCl (308 nm) excimer laser (Neev *et al.*, 1992). Many excimer

lasers, including KrF (248 nm), and nitrogen lasers (337 nm) function at a wavelength in the UV spectrum. Ultraviolet wavelengths are close to the absorption wavelength of DNA (260 nm). As a result, these lasers are minimally used in reproductive technologies due to concern for mutagenic effects (Green *et al.,* 1987**;** Hammadeh *et al.,* 2011; Kochevar *et al.,* 1989).

The next generation of lasers were designed to circumvent dangers of UV wavelength and cytotoxicity by emitting wavelengths in the infrared region (>800 nm) (Ebner *et al.,* 2005). The first of the newer generation of lasers to be used in IVF was the 2.9 um pulsed erbium:yttrium-aluminum-garnet laser (Er:YAG) (Feichtinger *et al.*, 1992). This device's use is limited by the need for constant contact with the embryo, as well as limitations due to interactions with the liquid media (Rink *et al.,* 1996). The next development was the holmium:yttrium-scandium-gallium-garnet laser (Ho:YSGG) with 2.1 um emission. In order to retain the beneficial effect of the infrared emission wavelength with this laser, the embryos require additional manipulation on a quartz slide, offsetting the advantages obtained by a safer wavelength (Schiewe *et al.,* 1995).

Currently, the 1.48 um diode wavelength indium-gallium-arsenic-phosphorus (InGaAsP) semiconductor laser is used in IVF. It is a non contact laser, has a safer wavelength and produces consistent results in the form of uniform, smooth edged tunnels (Rink *et al.,* 1996). This diode laser is delivered through a complex arrangement, requiring 3 mirrors and 3 lenses. A continuous laser beam is emitted and collimated by a microscope objective, and then paired with a visible beam. These pass through a mirror which reflects the invisible beam and is partially transparent to the 670 nm wavelength. Both beams are then directed through the primary microscope objective lens and to the desired object. The variability is less than 1 um, showing excellent reproducibility. Use of this laser does not require additional manipulation of the embryo or pose threat to DNA integrity by damaging radiation (Rink *et al.,* 1996).

3. Laser characteristics for IVF

Lasers in IVF have a wide variety of applications, however, the desirable characteristics of the laser used are similar across those applications. During laser targeting, the embryo's unique culture environment must remain consistent at all times to optimize the potential for a viable pregnancy. To that end, any laser used in the IVF laboratory must be very precise, extremely consistent with reproducible results and integrate well into the equipment required for routine IVF. In addition, it must not pose any additional threat to the integrity of the embryo. This includes an infrared wavelength to avoid direct chromosomal damage. It also helps when a non-contact mode is employed to avoid any unnecessary manipulation of the fragile embryo. Contact mode lasers requiring glass pipettes (UV wavelength) or quartz fibers (infrared wavelengths) add a layer of complexity with respect to additional manipulation of the embryo (Hammadeh *et al,* 2011). Similarly, no additional changes or alternations of media should be made to avoid undue stress on the embryo's environment, which should be kept at a physiologic pH of 7.2 and at 37 degrees Celsius at all times to optimize growth (Douglas-Hamilton & Conia, 2001 as cited in Al-Katanani *et al.,* 2002). This limits use to lasers which will not produce a thermal effect on the media containing the embryo, which is impacted by the laser's power, number of shots required, pulse length and irradiation time. Ease of use and speed of a technique also contribute to maintaining an appropriate environment for the embryo in that a faster procedure exposes the embryo to a hostile environment for a much shorter period of time.

Lasers have three characteristics directly impacting embryos: wavelength, power and pulse length. Wavelengths used in IVF tend to remain above 750 nm, in the infrared region, to avoid mutagenic effects on DNA (Kochevar *et al.*, 1989; Taylor *et al.*, 2010). The amount of power in a single laser remains constant but impacts the diameter of the hole created as well as the amount of heat emitted in the process, with higher power translating to larger diameter and increased heat (Taylor *et al.*, 2010). Different lasers may each have a different power. A similar scenario exists with pulse length, which can vary from 20 ms to >1,000 us. A longer pulse length also correlates with a larger hole (Rink *et al.*, 1996). Focusing the beam waist on a target provides a larger diameter of tunnel as well (Neev *et al.*,1992).
Beyond the physical characteristics of the laser itself are secondary characteristics and limitations impacting embryo use. For example, the mineral oil overlay may adhere to optical fibers in a contact mode laser, absorb additional heat and thus expand, moving the embryo and disrupting the path of the laser beam (Neev *et al.* 1992). The optical fibers used must be sterilized, as well as the micropipette tips, expensive disposable equipment leading to increased costs. Additional instruments used for manipulation introduce increased cost and possible damage to the embryo in the form of contamination and constant physical contact.

4. Applications of laser in IVF

Since the discovery of laser in 1960s, it has found application in many fields. The accuracy, versatility and spatial focusing potential have helped it to find a wide application in the

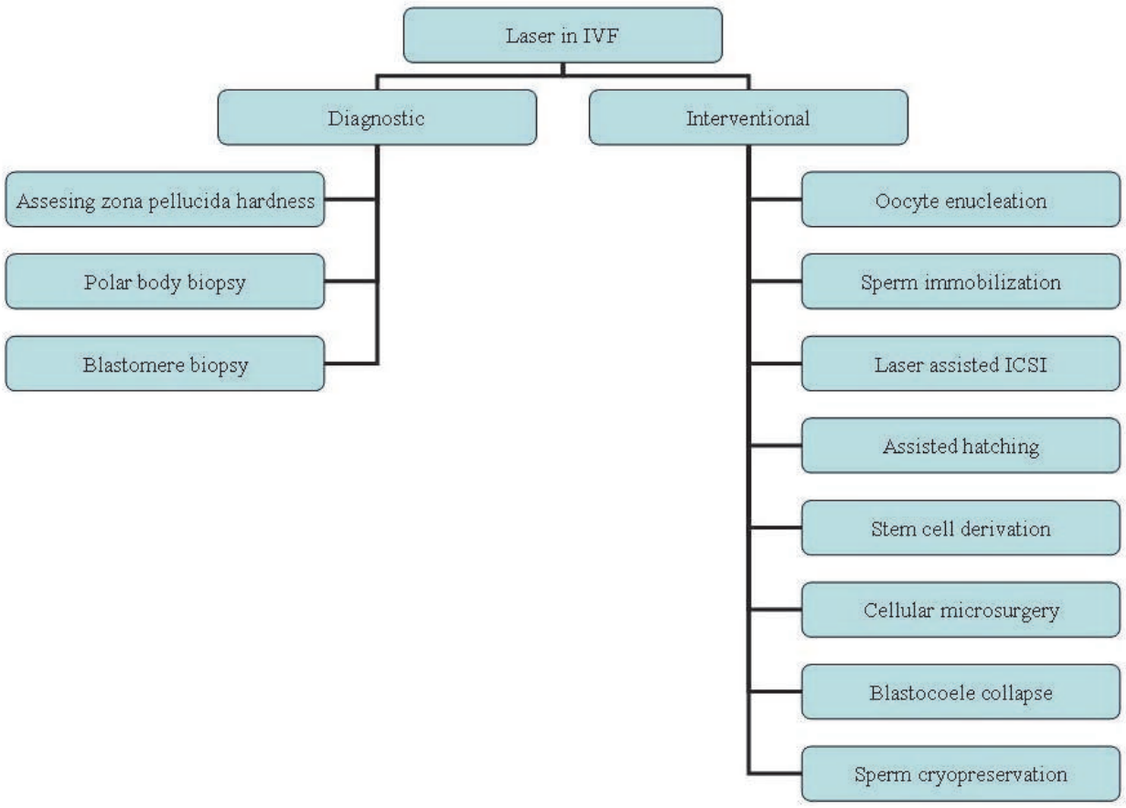

Fig. 2. Applications of lasers in IVF

medical arena. The applications of laser in IVF may be classified into diagnostic and interventional use for the ease of discussion (Figure 2). Diagnostic techniques include assessing the strength of the zona pellucida and pre-implantation genetic diagnosis. Interventional or therapeutic techniques involve manipulating individual gametes with oocyte enucleation and sperm immobilization, aiding fertilization and development with laser assisted ICSI and assisted hatching. Additional material may be obtained with stem cell derivation and cellular microsurgery. Embryos are optimized for freezing with blastocoele collapse. Regardless of the specific procedure, lasers provide an excellent method for precise intracellular surgery (Raabe *et al.,* 2009).

4.1 Diagnostic techniques

4.1.1 Assessing the zona pellucida

The zona pellucida is the hard protein coat surrounding and protecting the genetic material carried within the egg. This layer is approximately 15-20 um thick and must be breached in order for the sperm to make contact with the egg. In vivo, entry of the sperm initiates a reaction to ensure no other sperm obtains access to the egg and further hardens the protein layer to protect the zygote as it travels to the uterus. The proteinaceous coating must ultimately thin to allow the embryo to break out of the shell and implant in the uterine lining, or endometrium. Studies using laser pulses have determined the extent to which the zona hardens during the period from oocyte to blastocyst (Montag *et al.,* 2000b) and further identify which embryos may need assistance with sperm entry or hatching. Zona hardness is greater during in vitro culture as compared with in vivo growth. Montag et al. (2000b) and Inoue & Wolf (1975a) have shown that identical laser pulses create larger holes ranging from 13-17 um in the zona at earlier stages (oocyte, zygote) as compared to more advanced stages of development (morula, blastocyst) where holes are smaller at 10-13 um. Also, larger holes were created in blastocysts cultured in vivo when compared with in vitro grown blastocysts, suggesting zona hardening during culture (Montag *et al., 2000b*; Rink *et al.,* 1996).

4.1.2 Pre-implantation genetic diagnosis

Pre-implantation genetic diagnosis (PGD) is the analysis of genetic material from the developing embryo prior to transfer to the uterus. This can be done on the oocyte/zygote by extracting a polar body or on the 8-cell embryo by extracting a single cell or blastomere. Once genetic material has been obtained it may be analyzed for genetic abnormalities. Screening of oocytes and embryos for common chromosome abnormalities, such as trisomy 21, can improve pregnancy rates and reduce miscarriage rates. Some couples may be interested in screening for specific genetic problems typically severe or lethal conditions, carried by one or both partners, in order to avoid having an affected child.

4.1.2.1 Polar body biopsy

During oocyte maturation to the metaphase II stage and also after fertilization, duplicated genetic material is extruded as polar bodies. The polar body can provide helpful information by reflecting the maternal genetic material contained in that egg. (Clement-Sengewald *et al.,* 2002; Verlinsky *et al.,* 1990). Abnormal oocytes with genetic defects can be selectively excluded (Clement-Sengewald *et al.,* 2002). Genetic assessment of the unfertilized egg permits women who would not consider discarding an affected embryo due to personal beliefs to be screened for age related aneuploidy or hereditary chromosomal defects. It may

also be performed in countries where it is illegal to perform blastomere biopsy to genetically screen embryos (Dawson *et al.,* 2005; Clement-Sengewald *et al.,* 2002; Montag *et al.,* 2004).
The polar body is located in the perivitelline space directly under the zona pellucida and outside of the oocyte. It can be extracted by traversing the zona. Prior to the introduction of lasers, biopsy was typically done by degradation of the zona pellucida with Tyrode's acid, after which a capillary tube would be used to aspirate the polar body. This technique was highly variable, led to inconsistent opening size and could easily lead to further damage or loss of cells. It also requires changing culture media and increasing the risk of contamination. Alternatively to acid, mechanical biopsy could be performed with sharp glass instruments, again introducing possibility for structural damage or alteration during the manipulations (Clement-Sengewald *et al.,* 2002; Dawson *et al.,* 2005; Ebner *et al.,* 2005). Regardless of the method used, the oocyte must remain intact to continue development and the polar body must allow adequate, undamaged material for genetic analysis.
When polar body biopsy is performed using lasers, a pulse is directed at the region of zona pellucida nearest the polar body. In a description by Montag *et al*. (1998) two pulses of 14 ms are given by a 1.48 um non contact laser, creating an opening of approximately 14-20 um. The material is then extracted with a blunt capillary, avoiding potential damage to the oocyte with a sharp instrument, and the entire procedure is completed in just a few minutes (Montag *et al.,* 1998). A similar procedure has been described by Clement-Sengewald *et al*. using a nitrogen 337 nm laser and a Nd:YAG laser (Clement-Sengewald *et al.,* 2002). That same group described extraction of the polar body using optical tweezers (Nd:YAG, 1064 nm) and laser (nitrogen, 337 nm) pressure catapulting to collect the polar body, further eliminating a source of contamination by introduction of another pipette. To catapult the polar body, it was mounted to a membrane on a slide with the inner cap of a microfuge tube placed next to it. One pulse of the laser was aimed at the membrane, freeing it to catapult onto the nearby tube cap (Clement-Sengewald *et al.,* 2002; Schutze & Lahr, 1998). Oocyte recovery rates were only 67% in humans following this complete laser extraction method. An improved blastocyst survival rate was noted when access was obtained via laser as compared with acid solution, further strengthening the argument for laser use (Dawson *et al.,* 2005).

4.1.2.2 Blastomere biopsy

Blastomere biopsy is similar to polar body biopsy in that both techniques require careful extraction of genetic material from a very delicate structure followed by genetic screening. This procedure is also performed to facilitate selection of the embryo most likely to establish a viable pregnancy with healthy offspring. Blastomere biopsy becomes relevant at a later stage in development, after fertilization. Couples opt for this technique typically when one or both parents carry a hereditary genetic defect they want to avoid passing to children (Vela *et al.*, 2009) or in cases of advanced maternal age to screen against aneuploid embryos.
Until the introduction of laser assisted opening of the zona, blastomere biopsy was performed by zona drilling with an acid tyrodes solution (Talansky & Gordon, 1986, as cited in Malter & Cohen, 1989). The embryo is immobilized and held in place while acid in a microcapillary tube is gently blown against the zona until it starts to dissolve. The acid is then aspirated and the embryo is quickly rinsed to remove traces of acid. The technique requires speed and expertise so as not to injure the embryo. The hole size can often be variable.

The procedure for a blastomere biopsy using laser is similar to PGD with a polar body. Laser pulse(s) are utilized to create a hole in the zona pellucida, through which a blastomere is removed (Taylor *et al.,* 2010). Analysis of laser pulse length in generating a hole for blastomere extraction showed longer pulse duration (0.604 ms vs. 1.010 ms) produced larger hole sizes (10.5 nm vs. 16.5 nm, respectively) (Taylor *et al.,* 2010). However, Taylor *et al*. found no difference in number of blastomeres lysed for a given pulse duration. They did find a difference in number of blastomeres required to be obtained in each group. The longer pulse duration group was noted to require additional blastomere biopsy. These results were impacted by half of the affected embryos originating from the same patient with poor quality embryos and cannot clearly be attributed to laser use.
Studies comparing embryos after laser assisted biopsy to untreated embryos showed no adverse effects of treatment and similar hatching and development rates (Joris *et al.,* 2003). When performed with human embryos, pregnancy rates after laser blastomere biopsy are comparable to mechanical blastomere biopsy (Schopper *et al.,* 1999). Comparison of blastomeres obtained during acid and laser mediated biopsies showed laser biopsy generated more intact blastomeres (Joris *et al.,* 2003).

4.2 Interventional techniques

4.2.1 Laser assisted ICSI

With male factor infertility, it is often necessary to assist fertilization by directly injecting a single sperm in to the oocyte, a technique known as Intracytoplasmic Sperm Injection (ICSI). The limited number of viable or motile sperm decreases chances of fertilization and a successful pregnancy using the conventional oocyte insemination technique. ICSI is performed by aspirating a sperm into a sharp glass needle (5 um in diameter), perforating the oocyte's zona and depositing the sperm into the ooplasm (Palermo *et al.,* 1992). Deformation of the oocyte during the injection process can trigger oocyte degeneration either as a result of egg fragility or due to force required to traverse the membrane (Rienzi *et al.,* 2001, 2004; Abdelmassih *et al.,* 2002; Palermo *et al.,* 1996). Damage to the oocyte also occurs by disturbing the spindle apparatus, damaging the oocyte cytoskeleton, introducing harmful materials or by removal of cytoplasm during the injection procedure (Moser *et al.,* 2004; Hardarson *et al.,* 2000; Tsai *et al,* 2000; Dumoulin *et al.,* 2001).
Laser assisted zona drilling prior to ICSI can be used to increase the likelihood of successful fertilization (Palanker *et al.,* 1991). This may be done with a 193 nm ArF laser, which was shown to drill very precise holes without undesired damage to the zona pellucida (Palanker *et al.,* 1991). A 1.48 um diode laser can also be used to assist with ICSI (Rienzi *et al.,* 2001, 2004). A small channel of 5-10 um in diameter is drilled using low energy pulses of less than 2 milliseconds duration, taking care to leave the innermost layer of zona intact. The ICSI injection pipette is introduced through this channel to deliver the previously immobilized sperm (Rienzi *et al.,* 2001, 2004; Abdelmassih *et al.,* 2002). Prior to laser assistance, this technique was limited by operator skill and a non standardized tunnel size, potentially leading to polyspermy or loss of genetic material (Rink *et al*, 1996). Laser assisted ICSI provides a less traumatic method to create an opening in the zona pellucida for the purpose of sperm microinjection, leading to decreased breakdown of oocyte membrane (5% vs. 37%, Abdelmassih *et al.,* 2002) and increased oocyte preservation, 97% vs. 85%, after ICSI (Rienzi *et al.,* 2004). The type of laser used is in infrared range and is not absorbed by nucleotides and is considered safer than its counterparts (Ebner *et al.,* 2005; Kochevar *et al.,* 1989). The decreased force necessary in penetrating the egg with the ICSI needle in entry may also

preserve embryo quality (Rienzi *et al.,* 2001**;** Nagy *et al.,* 2001) and has been shown to improve embryo quality and survival, even when using poor quality oocytes (Abdelmassih *et al.,* 2002). To ensure even less traumatic manipulation, sperm may be injected into the oocyte through a laser drilled hole using optical tweezers to achieve fertilization (Clement-Sengewald *et al.,* 1996, 2002).

Ultimately, to establish a pregnancy the embryo must "hatch" out of zona and implant on the uterine wall. A potential drawback to laser assisted ICSI is that the thinning of the zona may result in duplicate hatching sites. This allows the embryo to escape via two openings, resulting in either degeneration or twinning. The theoretical concern is that the embryo would hatch through the site created during assisted hatching but also through the ICSI site as well (Abdelmassih *et al.,* 2002). Moser *et al.* (Moser *et al.,* 2004) discovered thinning the zona pellucida instead of completely opening it eliminated the concern for a second opening and incidentally improved blastulation rates through that site as well.

4.2.2 Sperm immobilization & selection

Sperm immobilization is critical when performing ICSI. The beating of the sperm tail in the oocyte after injection can cause damage. Typically during ICSI, the sperm tail is positioned under the glass microcapillary injection needle. The needle is brought down and across the tail causing it to break and immobilizing the sperm (Palermo *et al,* 1992; Nijs *et al*, 1996; Vanderzwalmen *et al.,* 1996; Yanagida *et al., 2001*). Fertilization rates are also closely linked to sperm immobilization, increasing from 54% to 68% (Vanderzwalmen *et al.,* 1996). Disruption of the sperm membrane aids the release of sperm factors important in oocyte activation (Dozortsev *et al., 1997).* Low level laser pulse can also be used to immobilize sperm, without affecting viability (Montag *et al.,* 1998**,** 2000d, 2009; Rienzi *et al,* 2004; Tadir *et al.,* 1990).

A rather unique application of laser is to identify and select viable sperm for ICSI. Usually motility is used as an indicator of living sperm. However in severe male factor cases such as asthenozoospermia, no motile sperm may be evident. This makes it very difficult to identify and select viable sperm for ICSI. A single laser pulse applied at the tip of a sperm's tail can aid in distinguishing living non-motile sperm from dead sperm. The tail of a viable sperm will curl, whereas the nonviable sperm will not respond to the laser pulse. Fertilization rates would be expected to be correspondingly higher if better sperm are selected for the injection (Montag *et al.*, 2000d, 2009). An alternative method for manipulating sperm includes optical trapping. Optical trapping uses a single beam non contact laser to move sperm during after immobilization or during ICSI (Clement-Sengewald *et al.*, 1996, 2002; Tadir *et al.,* 1991). The optical tweezers can hold actively moving sperm and determine their velocity (Clement-Sengewald *et al.*, 2002; Tadir *et al.,* 1991). Lasers used in optical trapping may be either infrared or ultraviolet (Clement-Sengewald *et al.*, 2002; Tadir 1989. Advantages of this technique include ease, no requirement for sophisticated micromanipulation skills or additional expensive disposable equipment. The capacity for the optical tweezers to determine velocity permits studies of medications on motility (Tadir *et al.,* 1989). It may also be used for polar body extraction or chromosomal manipulation (Tadir *et al., 1991).* Disadvantages include increased exposure time of the embryo to lasers, possible ultraviolet exposure depending on wavelength utilized and a potential adverse effect on the sperm (Tadir *et al.,* 1989).

4.2.3 Assisted hatching

To establish a successful pregnancy, the developing embryo must break out of its shell (zona pellucida) on day 5 or 6 by a process known as hatching. Once the embryo is hatched, it may implant on the endometrium and begin to grow but if it is unable to hatch, the pregnancy will not continue. Various factors contribute to failed hatching and implantation – increased maternal age, decreased egg quality, poor embryo and zona morphology to name a few, and the exact cause of failed hatching is unknown (Balaban *et al.,* 2002). An increase in zona hardness has also been implicated during in vitro fertilization (Inoue & Wolf, 1975; Montag *et al., 2000;* Balaban *et al.,* 2002**)**. The physiologic mechanism leading to hatching is likely different in vivo than in vitro, with in vitro embryos hatching when a critical cell number has been reached. This is compared with hatching independently of cell mass in vivo, likely related to lytic enzymes found in vivo (Montag *et al.,* 2000a). It has become relatively common practice to facilitate the hatching of blastocysts by creating an artificial opening in the zona pellucida either by mechanical, chemical or optical methods, although the exact population benefiting most from this procedure is yet to be determined (Hammadeh *et al.,* 2011). Assisted hatching has been proposed to be potentially more beneficial in patients over 40, with thicker zonae or poor prognosis patients (Balaban *et al.,* 2002; De Vos & Van Steirteghem, 2000; Hammadeh *et al.,* 2011; Sagoskin *et al.,* 2007; Lanzendorf *et al.,* 1998).

In the late 1980s, Cohen *et al.* mechanically opened the zona pellucida, achieving higher implantation rates. Since that time, multiple methods have been proposed to facilitate hatching (De Vos & Van Steirteghem, 2000; Cohen *et al.,* 1990). Zona drilling uses Tyrode's acid solutions to create a defect in the zona (Malter & Cohen 1989; Ebner *et al.,* 2005; Neev *et al.,* 1992; Balaban *et al.,* 2002; De Vos & Van Steirteghem, 2000), whereas mechanical hatching utilizes a microneedle to slice off a thin piece of the zona (Malter & Cohen 1989**;** Ebner *et al.,* 2005; Balaban *et al.,* 2002; De Vos & Van Steirteghem, 2000). Enzymatic hatching using pronase to generally thin the zona pellucida is also an accepted method of assisted hatching (Balaban *et al.,* 2002; Fong *et al.,* 1998). Direct comparison of hatching methods is challenging due to inter-operator variability, differing depths of zona penetration and heterogeneous patient populations.

Laser provides an alternate means to facilitate hatching, and is faster and easier than other methods (Balaban *et al.,* 2002). The 2.94 um Er:YAG laser has been used for assisted hatching with a significant increase in pregnancy rates (Antinori *et al.,* 1996). The laser was deemed safe for clinical use after trials in animal models (Obruca *et al.,* 1994, as cited in Obruca *et al.,* 1997). The 1.48 micron infrared diode laser beam has been more widely used in clinical IVF labs as an efficient and simple method for embryo hatching. Multiple studies have demonstrated its safety (Sagoskin *et al.,* 2007; Lanzendorf *et al.,* 2007; Wong *et al.,* 2003) as well as efficacy when compared to acid hatching (Lanzendorf *et al.,* 2007; Balaban *et al.,* 2002; Jones *et al.,* 2006).

The optimal technique for laser assisted hatching is still being debated. The laser can be used to thin a large area of the zona, partially hatch by creating an incomplete hole or completely hatch by drilling completely through the zona (Figure 3). The number of shots and duration of pulse exposure is also subject to discussion with investigators varying parameters to achieve an appropriate tunnel size. Optimal hole size is as yet unclear, although >10 um leads to improved results (Ebner *et al.,* 2005). A study by Montag *et al.,* found no evidence of impaired growth or adverse effects as a result of laser hatching (Montag *et al.,* 2000a). Advocates of partial hatching argue increased safety using this

method because the laser does not come in to direct contact with the embryo. Finally, proponents of the zona thinning technique contend that overall thinning will avoid inadequate hatching and be more likely to correspond with the natural hatch site due to a larger area being ablated (Moser *et al.,* 2004). Studies comparing multiple methods of hatching yield inconclusive results and no definitive recommendations can be made. A study comparing pulse intensity and number of pulses determined 50% intensity with 2 pulses was the optimal setting to increase blastocyst formation (Tinney *et al.,* 2005) by creating a complete hole rather than the less effective zona thinning. Specific settings to achieve those results would be expected to vary based on the power of different lasers. Mantoudis *et al.,* 2001, compared the three methods of laser hatching and determined partial hatching or thinning the zona is more effective. Implantation rates were 2.8%, 9.1% and 8.1% in the complete hatching, partial hatching and zona thinning groups. Clinical pregnancy rates were also significantly improved with 5.2%, 18.3% and 22.1%, respectively. Thinning in this study ablated the zona around 25% of the embryo, leaving only the inner membrane of the zona pellucida intact in that section. It is unclear what the diameter of the complete hatch site was in this study. Another concerning trend in this study was 22% of pregnancies were multiple pregnancies, more than typically seen (Mantoudis *et al.,* 2001), which is not unique to this trial (Hammadeh *et al.,* 2011). In contrast to the findings of Mantoudis *et al.,* Wong *et al.* found improved hatching rates with complete hatching compared to partial hatching, 38% vs. 25%, respectively (Wong *et al.,* 2003). Laser-assisted zona pellucida thinning prior to ICSI resulted in decreased oocyte degeneration rates, better blastocyst hatching rates and improved pregnancy rates after day 3 embryo transfer (Moser *et al.,* 2004). In this study embryos had their zona pellucida thinned by 50% via 5-6 laser pulses, covering at most 70 um of zona. A trial by Balaban *et al.* compared assisted hatching by laser, acid Tyrodes, pronase treatment and mechanical technique. These investigators concluded that all methods were comparable based on the outcome parameters studied, including implantation and pregnancy rates, multiple pregnancy rates and abortion rates (Balaban *et al.,* 2002). Additional studies comparing laser assisted hatching with acid drilling showed no significant differences with respect to pregnancy rates (Lanzendorf *et al.,* 2007; 1999; Jones *et al.,* 2006).

Laser assisted hatching is generally well-accepted in IVF labs, allowing improved standardization between operators (Lanzendorf *et al.,* 2007; Jones *et al.,* 2006). Children followed to one year of age after an assisted pregnancy using laser assisted hatching were

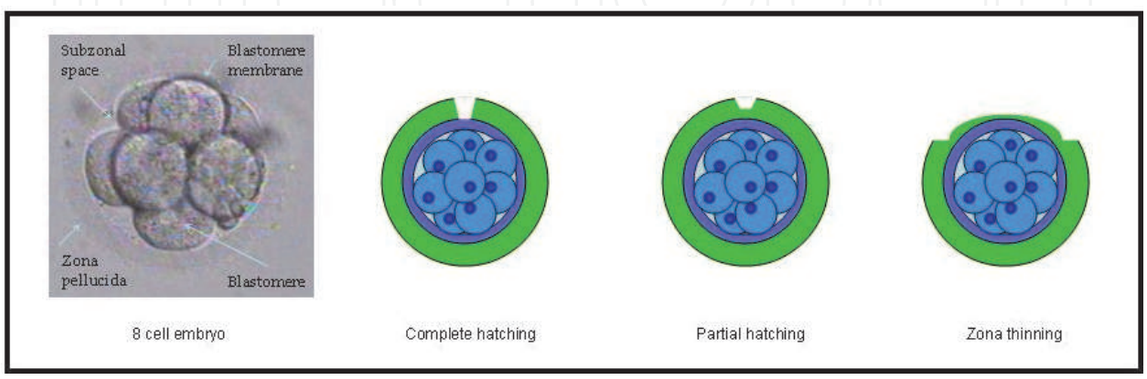

Fig. 3. Assisted hatching

found to have no increase in congenital malformations (Kanyo & Konc, 2003). Other pregnancies have also yielded healthy babies following laser assisted hatching (Lanzendorf *et al.*, 1998). The first 1.48 um laser to receive US FDA approval for clinical use in assisted hatching was the ZILOS-tk in 2004. This was followed by the Octax laser in 2006 and the Saturn Active Laser System in 2008.

4.2.4 Laser pulse blastocyst collapse

As the efficiency of embryo culture increases, supernumerary embryos are produced and cryopreserved for transfer in a future cycle (Iwayama *et al.,* 2010; Gardner *et al.,* 1998). One method of cryopreservation known as "vitrification" involves high molar concentrations of cryoprotectants and rapid cooling of the embryo at rates of -20,000 C°/min (Desai *et al.,* 2011). This cooling technique is extremely effective for embryos at all stages. The high cooling rate prevents ice crystal formation in cellular cytoplasm. Post-warming survival rates have been high with this technique. Yet it was observed that well-developed and expanded blastocysts had lower survival rates than the less mature blastocyst or the morula stage embryo (Vanderzwalmen *et al.,* 2002). The primary structural difference between the early stage blastocyst or morula and the later stage blastocyst is the presence of a fluid filled cavity in the expanded blastocyst, called a blastocoele.

Artificial shrinkage of the blastocyst to reduce fluid volume in the blastocoelic cavity before freezing was investigated as a technique to increase survival and ultimately increase clinical pregnancy and implantation rates (Vanderwalzmen *et al., 200*2). This has been carried out by either mechanical puncture of the blastocyst cavity with a needle and withdrawal of fluid (Vanderwalzmen *et al.,* 2002), use of osmotic shock to draw out fluid (Iwayama *et al.,* 2010) or by using laser pulses to collapse the blastocyst (Mukaida *et al.,* 2006) (Figure 4). In mechanical collapse, the inner cell mass of the blastocyst is positioned at 12 o'clock or 6 o'clock position. A glass micro needle is introduced into the cavity of the blastocoel and then withdrawn, which results in collapse of the cavity over 30 seconds to 2 minutes (Vanderwalzmen *et al.,* 2002; Mukaida *et al.,* 2006). During osmotic shock, the blastocyst is passed through media with high concentrations of sucrose to essentially "dehydrate" the embryo (Iwayama *et al.,* 2010). For laser collapse, a short duration laser pulse directed at the trophectoderm in a region away from the inner cell mass is delivered, shrinking the cavity immediately without additional manipulation of the embryo (Mukaida *et al.,* 2006). No statistical difference was seen on comparison of mechanical versus laser shrinkage (Mukaida *et al.,* 2006), or with osmotic versus laser shrinkage (Iwayama *et al.,* 2010), although results were improved in both cases as compared to controls (Mukaida *et al.,* 2006; Iwayama *et al.,* 2010). Human and mouse blastocsyts vitrified after mechanical or laser collapse have fewer damaged cells than untreated controls and total blastomere counts are higher after 24 hours of culture (Desai *et al.,* 2008). The rate of re-expansion after warming was also found to be higher (Desai *et al.,* 2008). In this study, an OCTAX 1.48 uM laser was used to deliver a single shot 10 ms pulse to the junction of cells located in the trophectoderm. The complete collapse of the blastocysts was seen within 2-4 minutes.

The major safety concern for use of laser is that the inner cell mass which ultimately becomes the fetus will inadvertently be exposed to the laser pulse. At this time the FDA has not approved this particular application of the laser in the U.S.

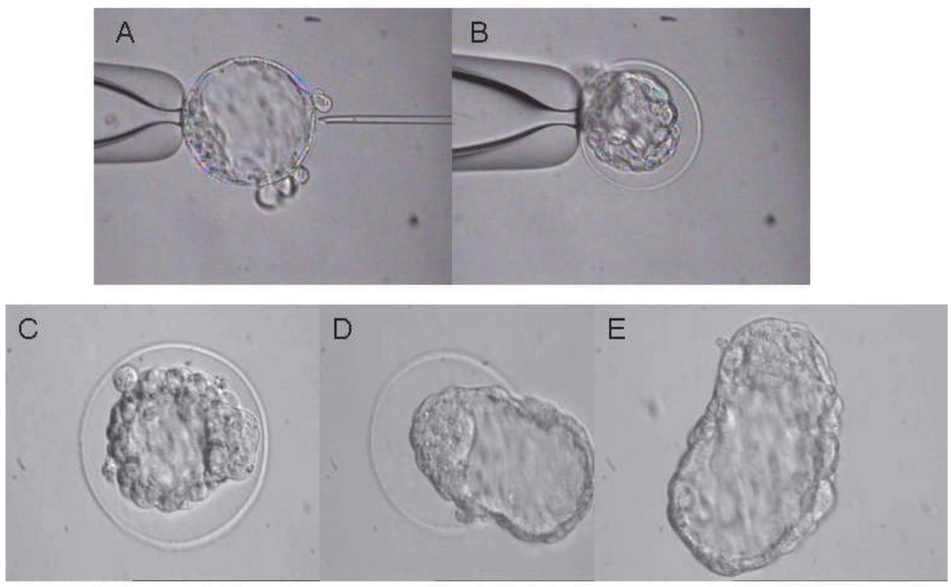

Fig. 4. (A) Blastocyst during mechanical collapse with ICSI needle. (B) Blastocyst immediately after collapse, (C) Blastocyst rewarmed after laser collapse, (D) 3 hours after rewarming, (E) after culture for 24 hours

4.2.5 Cellular microsurgery

Lasers may be used to remove material within the blastocyst that may prove detrimental to its development. This detrimental material includes cellular fragments or necrotic blastomeres. During embryo development it is possible to see cellular fragments appear. This is a process that may lead to impaired development as cells are dividing and natural planes are obstructed with fragments (Ebner *et al.,* 2005; Alikani *et al.,* 1999). Embryos with higher levels of fragmentation were found to have decreased implantation and pregnancy rates (Alikani *et al.,* 1999; Sathananthan *et al.,* 1990). Necrotic blastomeres are frequently observed in cryopreserved embryos upon warming. Release of toxic metabolites from dying cells may interfere with subsequent implantation. (Rienzi *et al.,* 2002). The laser can be used to create a small opening enabling extraction of fragments as well as dead cells. When necrotic blastomeres are removed, cleavage and implantation rates improve, and pregnancy rates increase from 17% to 45% (Rienzi *et al.,* 2002).

Another type of microsurgery that is well suited to the laser technology is preparation of zonae for the hemizona assay. The hemizona assay is used as a diagnostic tool to assess the binding capacity of sperm to the oocyte zona and also as a research model to study the effects of the environment or administered medications on the zona pellucida (Schopper *et al.,* 1999; Montag *et al.,* 2000c, 2009). For this procedure, the test oocyte is sliced into two sections, one to be used as the control and the other for the test treatment. A critical aspect of maintaining the accuracy of the test is that the oocyte is evenly divided so comparisons can be made. This bi-section can be accomplished using a mechanical technique or with the laser. Consecutive adjacent laser shots can be used to drill a series of holes through an oocyte immobilized using a micropipette (Montag *et al.,* 2000c). A study comparing laser to mechanical hemizona creation showed no difference in sperm binding between the two

methods, and the laser drilling produced very even, flat hemizonae (Montag 2000c). The hemizona assay is performed more easily using lasers via mechanical techniques with a microscalpel (Schopper *et al.,* 1999).

The laser is particularly well suited for cellular microsurgery. The introduction of a laser with a femtosecond pulse to be used as a laser scalpel may further increase the accuracy of diagnostic and interventional procedures performed on the embryo (Rakityansky *et al.,* 2011). Biopsy of the trophectodermal cells of the blastocyst for pre-implantation genetic screening is one possibility. Currently the ability to accurately deliver the laser pulse to a very fine area and minimize heat transfer to adjacent cells has been a concern, limiting this use of lasers to research. Lasers may also be further developed to aid in elucidating a proteomic profile for embryos to help predict their success (Vela *et al.,* 2009).

4.2.6 Stem cell derivation

Embryonic stem (ES) cell lines are derived from the inner cell mass of blastocysts. Once isolated the inner cell mass can be used to establish pluripotent stem cell lines for use in transplants and to study cellular differentiation (Turetsky *et al.,* 2008). The ICMs from embryos that have been diagnosed with genetic disorders after PGD screening are potential source material for developing cell lines containing specific genetic conditions (i.e. cystic fibrosis, hemophilia) for use in research. Mechanical dissection of the inner cell mass from the trophectoderm is highly operator dependant, and chemical dissolution of the trophectoderm with Tyrode's acid subjects the inner cell mass to possible damage from the corrosive fluid (Turetsky *et al.,* 2008). Removal of the inner cell mass using several laser pulses has been shown to be an effective and an easy method to extract this stem cell source material from the blastocysts to establish ES cell lines (Turetsky *et al.,* 2008; Tanaka *et al.,* 2006). Laser also facilitates ICM isolation for cryopreservation of the stem cell source material (Desai et al., 2011).

4.2.7 Oocyte enucleation

Oocyte enucleation is similar to the process of dissecting the inner cell mass away from the outer layer of cells in an embryo. It is more challenging in the sense that only one cell, the oocyte, exists rather than the many cells in a blastocyst. Enucleation separates the nucleus of the oocyte from the remaining cellular material, effectively removing all genetic potential from the oocyte (Hirata *et al.*, 2011). This is done to establish cell lines for research purposes and to explore the genetic reprogramming potential of the oocyte cytoplasm (Hirata *et al.,* 2011; Malenko *et al.*, 2009; Raabe *et al.,* 2009). Once the nucleus with chromosomes is removed using a micropipette, new genomic material from somatic cells is introduced in to the enucleated oocyte (Malenko *et al.,* 2009; Hirata *et al.,* 2011). This may be done to develop embryonic cell lines for future therapeutic use (Hirata *et al.,* 2011). Using this procedure the cytoplasm of the oocyte reprograms differentiated somatic chromosomes into embryonic cells (Hirata *et al.,* 2011). Women who may feel uncomfortable donating eggs for research or therapeutic uses because the oocyte contains their genetic material may be more willing to donate knowing their genome will be removed (Hirata *et al.,* 2011).

The 1.48 uM diode laser may be used in conjunction with oocyte enucleation procedures in a similar manner as with ICSI and assisted hatching. A small hole is drilled in the zona pellucida, through which the nucleus is removed while leaving most of the cytoplasm (Li *et al.,* 2009). A picosecond pulsed 405 nm diode laser also effectively aids in enucleation with

extremely short pulse duration of 1-2 seconds. This laser has not been approved for use in humans, however it is of interest due to its effect on intracellular structures (Raabe *et al.,* 2009). Although intracellular organelles were not found to be directly harmed following irradiation, function during the cell division process was prolonged when compared to non-irradiated cells. This indicates non-specific damage may have occurred (Raabe *et al.,* 2009) and cautions for judicious study of non-specific effects of irradiation in human embryology.

5. Safety & regulations

Lasers are currently considered by the Food and Drug Administration as a Class II device, special controls. As a Class II device, lasers must go through more than the general control measures regarding marketing and safety standards. They do not, however, have the stringent requirements and prolonged approval process prior to marketing required of the more highly regulated Class III devices. Class III devices are considered to be high risk, to the level of supporting life or presenting an unreasonable risk of harm. Three lasers have been approved by the FDA for use in reproductive technology: Saturn Active Laser System, Octax Laser Shot System and Hamilton Thorne Zona Infrared Laser Optical System. These lasers have only been approved for ablation of a small hole in the zone pellucida or thinning of the zona pellucida in approved patients.

The use of lasers in reproductive technology, particularly with respect to embryology, has stirred numerous concerns since its initial application. Areas of concern focus on the safety of the procedure as related to embryos at the time of development and for the children those embryos ultimately become. Primary aspects of laser function related to this issue are wavelength, heat generation and direct injury to blastomeres or oocytes through additional manipulation or imprecise beams.

The wavelength of lasers in reproductive technology falls into either the ultraviolet or infrared spectrum. Those lasers that have ultraviolet wavelengths provoke concern for possible mutagenic damage to embryonic DNA. The peak absorption rate of DNA is at 260 nm. Any laser with a wavelength in the UV range of the spectrum, 10-380 nm, increases likelihood of genetic damage or cytotoxicity. This includes excimer lasers with wavelengths at 193 nm, 308 nm and nitrogen 337 nm (Clement-Sengewald *et al.,* 2002). Data collected after zona drilling on mouse embryos with a 1.48 um laser found no significant differences in DNA methylation or early gene expression (Peters *et al.,* 2009; Kochevar, 1989).

Thermal damage occurs with absorption of heat by media surrounding the cells of interest. This is particularly true of the Er:YAG laser, which has a wavelength in the infrared spectrum but may pose a threat to cells by elevating the temperature of the culture media while in use (Clement-Sengewald *et al.*, 2002). Cells subjected to elevated temperatures may produce heat shock proteins as a protective mechanism, particularly HSP70i. When produced, these heat shock proteins help to stabilize other proteins and prevent apoptosis (Al-Katanani & Hansen, 2002). In a study examining the production of heat shock protein after 1.48 um laser drilling, no increase in levels of HSP70i were noted. Of note, the embryos were exposed to larger doses of laser energy during experiments than during routine zona drilling (Hartshorn *et al.,* 2005). This lends credence to the belief that the 1.48 uM laser has no immediate adverse effects on the embryo as a result of heat generation. Additionally, embryos exposed to laser drilling continue to develop at the same, if not better, rates than control embryos, and thus do not exhibit the retardation of growth seen if a cell is heat shocked (Hartshorn *et al.,* 2005). An associated problem lies within optimal laser settings for

a given procedure and the differing damage sustained by two routes to the same objective. For example, although visible results and initial growth may be unchanged, the amount of thermal spread anticipated to emerge from a lower power but longer duration pulse is greater than a higher power but much shorter pulse (Taylor *et al.,* 2010; Tucker *et al.,* 2009). This could lead to abnormal development later due to thermal spread (Tucker *et al.,* 2009). Although the peak temperature is much lower when a low powered laser is used, the prolonged pulse time leads to more extensive heating of the media and cells within that media (Tucker & Ball, 2009; Taylor *et al.,* 2010). It is currently uncertain how this type of thermal spread affects outermost blastomeres. A study examining oocyte lysis, cytogenic development and oocyte development following polar body biopsy via laser determined no deleterious effects were seen after the procedure (Hammoud *et al.,* 2010).

Long term data on childrens' health after use of the 1.48 um diode laser for zona opening is still limited. A study by Kanyo and Konc (2003) found no increase in congenital malformations after this procedure which is quite reassuring. As the use of laser technology in reproductive medicine becomes more widespread, more long term studies will be needed to evaluate both congenital defects and DNA abnormalities that may not manifest until later in life.

6. Conclusions

Lasers are useful in IVF as an additional tool with which to perform delicate procedures. The most commonly used laser in clinical IVF labs is the 1.48 um diode laser. This laser appears to be relatively safe for polar body or blastomere biopsy, sperm manipulation, drilling through the zona pellucida, stem cell derivation and cellular microsurgery. Laser technology may make performance of these tasks faster and easier. Definitive recommendations regarding whether or not to use lasers in reproductive technology are lacking. No conclusive data exists regarding long term safety of laser assistance in reproductive techniques and should be investigated more closely in the future.

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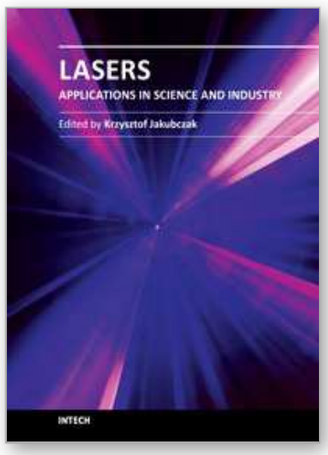

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The book starts with basic overview of physical phenomena on laser-matter interaction. Then it is followed by presentation of a number of laser applications in the nano-particles and thin films production, materials examination for industry, biological applications (in-vitro fertilization,tissue ablation) and long-range detection issues by LIDARs.

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