Chapter 1

Introduction: IVF concepts, embryo development, and embryo selection

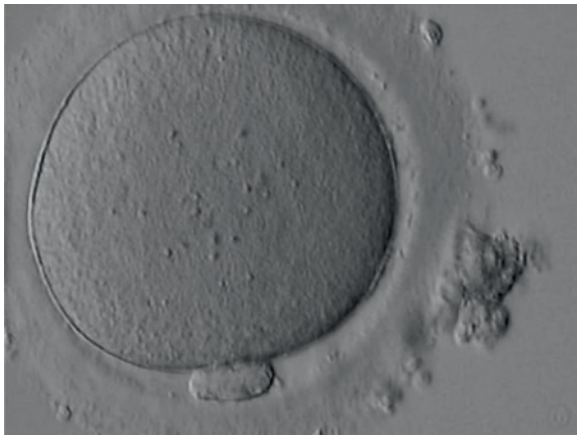
Thomas Ebner

1.1. Introduction

The birth of Louise Brown [1] represents a milestone in the history of assisted reproductive technologies (ART). While at the beginning of the ART era, treatment was only possible for patients showing normozoospermia or mild forms of male subfertility, severe male factor infertility could not be treated at all. In other words, conventional in vitro fertilization (IVF) worked well for female factor infertility, e.g., bilateral tubal blockage, but those patients with bad-quality semen samples or failed fertilization after IVF still had to face childlessness. This unwanted scenario led to intensive research in order to solve the problem of suboptimal sperm count and quality. What all the upcoming techniques had in common is the effort to bring the male gamete closer to the oocyte as routinely done in conventional IVF.

The first step was to breach the zona pellucida (ZP) and inseminate the collected cumulus-oocyte-complexes (COC). This approach, known as partial zona dissection or PZD [2], worked to a certain degree; however, the rate of polyspermic fertilization was rather high since the artificial opening in the ZP allowed bypass of the cortical reaction usually preventing the egg from polyspermy. The consequent next step was to place less sperm in the perivitelline space by subzonal injection (SUZI) [3]. Although this technique led to a reduction of polyspermy associated with a slight increase of regular fertilization, characterized by the presence of two pronuclei (2Pn), it was immediately replaced by intracellular sperm injection (ICSI) once Palermo and co-workers [4] placed a single spermatozoon into the ooplasm (Video 1.1), realizing that by doing so fertilization rates up to 70–80% could be observed while all drawbacks were excluded.

All of a sudden, all those male factor patients starting from oligo-asthenoteratozoospermia through to azoospermia were treatable. In the latter scenario several sites in the genitourinary tract could serve as a sperm source. Either spermatozoa could be aspirated from the epididymis [5], a technique called microsurgical epididymal sperm aspiration (MESA), or via TESE, which refers to a testicular biopsy [6].



Video 1.1 Intracytoplasmic sperm injection (ICSI). Inner diameter of ICSI pipette (MicroTech, Gynemed, Lensahn, Germany) is 5 µm.

1.2. Controlled ovarian hyperstimulation and ovarian puncture

Of course spermatozoa are only one side of the coin. The female counterpart can also be a limiting factor. In the early years of IVF exclusively natural cycles were used. Oocyte collection was routinely performed via laparoscopy. The combination of both approaches increased the risk of ending up without oocytes for further fertilization.

Time-Lapse Microscopy in In-Vitro Fertilization, ed. Marcos Meseguer. Published by Cambridge University Press.

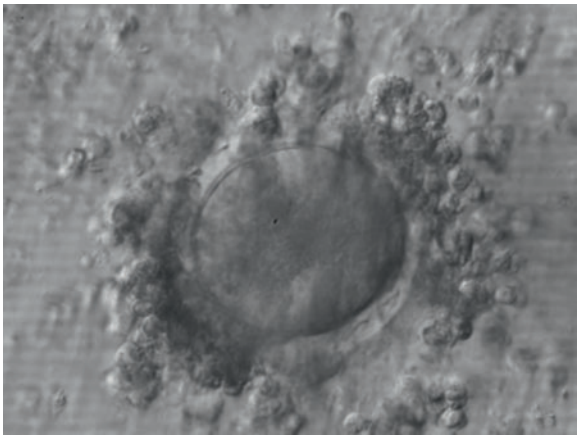
The development of transvaginal ovarian puncture [7, 8] and, particularly, of controlled ovarian hyperstimulation (COH) steadily increased the number of mature oocytes available for IVF or ICSI [9, 10].

In principle, to increase the number of follicles (and consequently of eggs) per ovary two main stimulation protocols are used. The first regimen is called "long protocol" since down-regulation of the pituitary gland using GnRH-agonists is started in the preceding cycle whereas ovarian stimulation applying human menopausal or recombinant gonadotrophins (LH and/or FSH) is done in the treatment cycle. The shorter antagonist protocol, on the other hand, represents a combination of gonadotrophin stimulation and immediate suppression of a potential LH surge with GnRH-antagonists, the latter being done if at least one follicle reaches the diameter of 12 mm and serum estradiol level appears adequate. Irrespective of the stimulation regimen, final maturation of the oocyte has to be performed by utilizing either human choriogonadotrophin (hCG) or a GnRH-agonist [11].

Based on hormonal parameters and follicular size oocyte retrieval is routinely carried out 36 hours after hCG application under ultrasound control. Where there is an indication of ovarian hyperstimulation syndrome ovulation induction can alternatively be done with a gonadotrophin agonist. However, the negative pressure exerted by the aspiration pump guarantees that the COCs located in the cumulus oophorus gather in the follicular fluid which is collected in special tubes. These vials then are handed over to the IVF laboratory where the embryologists separate the COCs from the sometimes hemorrhagic follicular fluid in order to transfer them in an optimized culture medium.

1.3. IVF

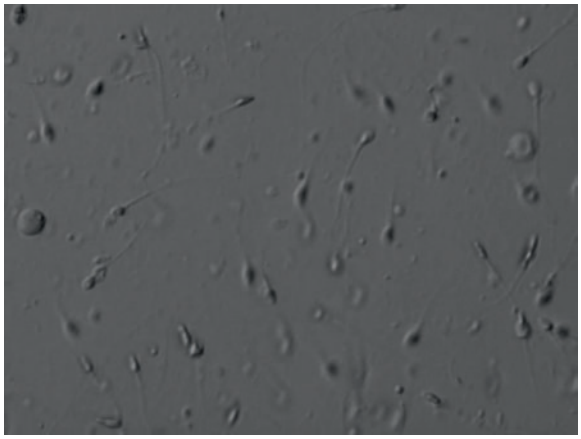
After a resting period of 2 to 3 hours, harvested COCs are evaluated by their appearance, particularly by the expansion of the corona radiata and the outer cumulus cells. Based on such criteria eggs within the cumulus matrix are roughly classified as either mature (metaphase II) or immature (pro- and metaphase I). In more detail, an expanded and/or luteinized complex and a radiant corona radiata indicate completion of nuclear maturation, while the absence of an expanded cumulus is associated with immaturity [12]. Recent publications, however, showed that nuclear maturation and oocyte quality cannot be predicted adequately by scoring the COC appearance [13, 14].



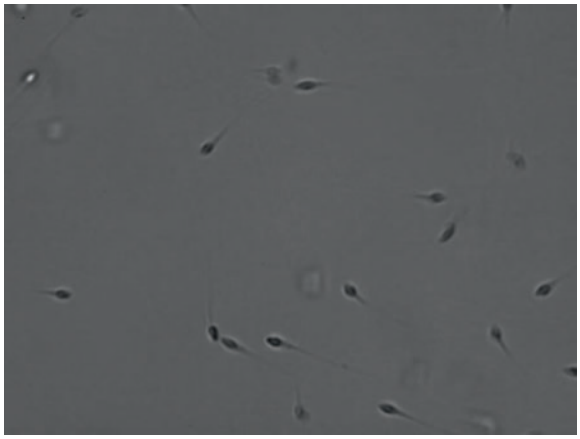
Video 1.2 In vitro fertilization (IVF).

Frequently, blood clots or other amorphous clumps [14, 15] are present in the COC. One tends to cut off these contaminated areas using needles but apart from the mechanical stress to the oocyte, this procedure is of no need since there is evidence that COCs showing blood clots have already been harmed during folliculogenesis and, thus, their developmental capacity may not be retained [14]. Thus, in the presence of a (borderline) normozoospermic sperm sample in conventional IVF (Video 1.2), COCs in their original state are inseminated with a sufficient number of spermatozoa, which turned out to be 25,000 to 50,000 per complex depending on the quality of the original ejaculate.

It is important to note that insemination is not performed with raw semen (Video 1.3), rather the sample is processed with different preparation techniques such as swim-up, mini swim-up or density gradient centrifugation, to name but a few. Since these processing methods include one or two centrifugation steps which could potentially harm the spermatozoa sperm selection chambers have been developed which avoid the above mentioned stressor. In such chambers spermatozoa accumulate by motility (Video 1.4) and as a collateral benefit contact between the gametes and seminal plasma (e.g., containing erythrocytes or inflammatory cells etc.) is kept at a minimum.



Video 1.3 Native ejaculate.



Video 1.4 Same semen as in Video 1.3 processed with sperm selection chamber (swim-over time 30 minutes).

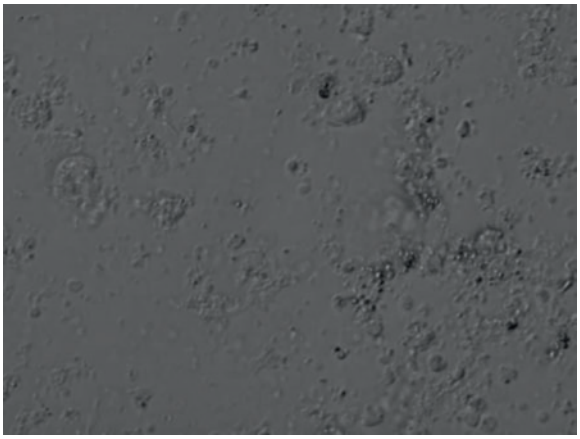
1.4. ICSI

For successful ICSI it is critical that the majority of cumulus cells are removed from the female gamete. Apart from technical problems, such as incorporation of foreign somatic DNA [16], control of oocyte maturity and quality can only be checked after proper denudation [17]. Stripping of the oocytes is traditionally achieved using enzymatic digestion of the outer matrix followed by mechanical denudation through pipetting. Since it has been shown that a dislocation between the first polar body and the meiotic spindle can occur if the mechanical part is performed inadequately, e.g., using pipettes of an inappropriate inner diameter, in routine work, hyaluronidase, which is the enzyme degrading hyaluronic acid (the major component of the extracellular matrix of oocytes), is used at the beginning of the denudation process. Most of the commercially available products have a concentration of 80 IU/l, which is only a tenth of the critical threshold above which parthenogenetic activation does occur [18]. Alternatively, plant or recombinant human products can be utilized [19, 20].

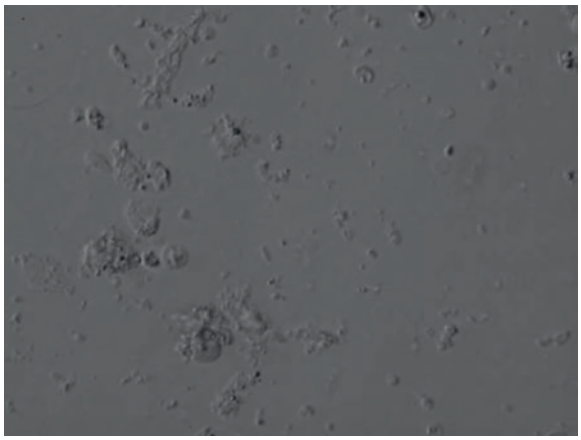
Once the ejaculate is processed in order to separate motile from non-viable sperms, an a priori motile spermatozoon is selected, which has to be immobilized prior to injection [21]. Immobilization of the sperm in ICSI has two beneficial effects: on the one hand, any theoretical damage to the cytoskeleton caused by motile sperms is negligible, and on the other, permeabilization of the sperm membrane will ensure that phospholipase C zeta, a sperm-derived oocyte-activating factor, immediately enters the ooplasm, thus initiating oocyte activation [22]. Sperm immobilization is usually performed towards the end of the tail (Video 1.5); however, permeabilizing other sites is an alternative [23]. It can be performed using either mechanical [21, 24] or piezoelectrical manipulation [25, 26]. Recently, a laser-assisted permeabilization technique (Video 1.6) was introduced into the field of ICSI [27–29]. In detail, male gametes were immobilized with a non-contact diode laser applying two successive laser irradiations per spermatozoon, the first aimed near the middle of the tail and the

Video 1.5 Mechanical immobilization as in a single sperm.

Video 1.6 Laser-assisted immobilization of a single sperm. Laser shot can be divined in the middle of the image section.



Video 1.7 Fresh testicular biopsy showing one non-progressive motile sperm with residual body at the end of the video.



Video 1.8 Same biopsy after cryopreservation. Restoration of progressive motility has been done with a ready-to-use theophylline (GM501 SpermMobil, Gynemed, Lensahn, Germany).

second directly at the end of the tail. This strategy minimized the total energy dose male gametes were exposed to.

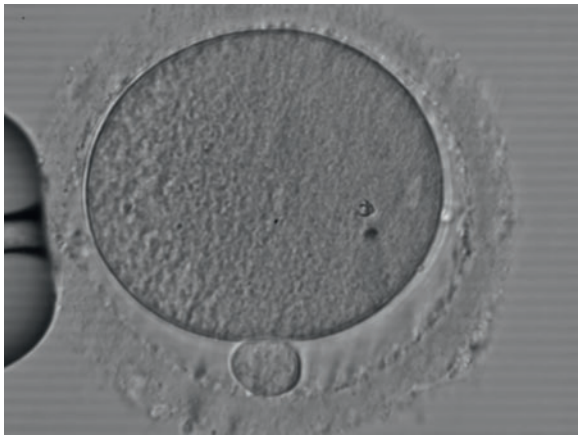
Regardless of the source of the sperm, ICSI is done according to a standardized procedure. The oocyte is held in place with a holding pipette at the 9 o'clock position (provided that the first polar body is located at the 6 or 12 o'clock position). Once the equatorial plane of the oocyte is in focus, the ICSI-pipette has to be pressed against the zona pellucida (3 o'clock) creating a characteristic funnel. After penetrating both the zona and the oolemma, a small volume of cytoplasm should be aspirated into the glass tool to activate the egg and to ensure rupture [30]. A single permeabilized spermatozoa is then gently placed near the horizontal axis. Withdrawal has to be done carefully to prevent leakage from the oocyte.

Sometimes, e.g., in case of cryopreserved or testicular sperm, embryologists have to deal with exclusive presence of immotile sperm (Video 1.7). This scenario bears the risk of selecting non-viable spermatozoa if viability is not estimated with either an elasticity test [31], a hypo-osmotic swelling test [32], or by the usage of laser pulses [33]. Most elegantly this viability check is done by the help of dimethylxanthines, which allow for partial restoration of original motility [34] (Video 1.8).

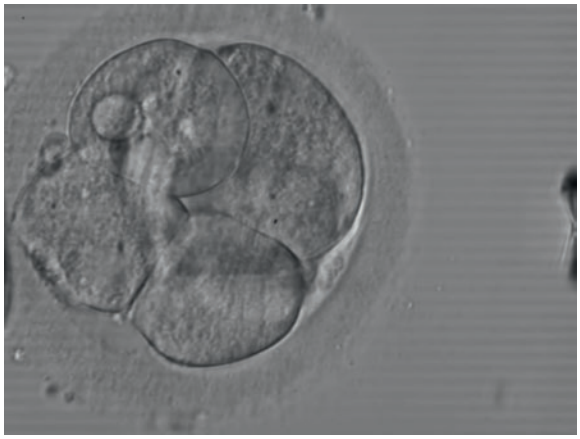
The most important prerequisite for a best prognosis ICSI performance is a well-calculated time schedule. With respect to this, the period between oocyte collection and subsequent ICSI should never exceed 6 hours [35, 36] in order to avoid in vitro aging of the oocytes. However, it is rather irrelevant whether the COCs are denuded immediately after collection (e.g., leaving denuded eggs in culture for later ICSI) or if the manipulation is performed directly prior to ICSI after a resting period of several hours [35].

1.5. In vitro culture

Immediately after IVF or ICSI, the inseminated COCs or injected eggs, respectively, are transferred to special culture dishes for prolonged in vitro culture (up to blastocyst stage if possible). Primarily, two



Video 1.9 Biopsy of first polar body. Zona opening is done with a diode laser at the 5 o'clock position. Inner diameter of biopsy pipette (MicroTech, Gynemed, Lensahn, Germany) is 15 µm.



Video 1.10 Blastomere biopsy in a donated embryo showing a large blastomere with binucleation (out of focus). It should be noted that the embryo has already started to compact. Inner diameter of biopsy pipette (MicroTech, Gynemed, Lensahn, Germany) is 30 µm.

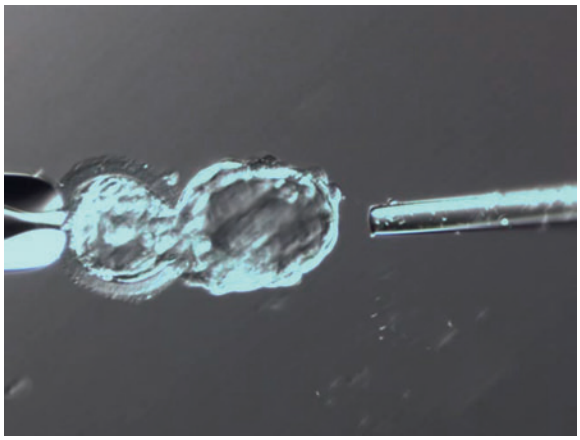
parameters influence the yield and quality of blastocysts out of a pool of fertilized oocytes.

Firstly, embryologists have to decide which type of culture medium they want to use. Advocates of the "back to nature" principle would rely on sequential culture media whereas followers of the "let the embryo choose" philosophy would rather use a global medium. The main difference between these types is that sequential media are composed of two different media in order to mimic the milieu in the oviduct as well as in the uterus. Global media are more universal and can be used from the very beginning up to blastocyst stage. Meanwhile it turned out that both concepts will result in a comparable number of viable blastocysts [37].

Embryo density is the second parameter that might positively affect blastocyst outcome. In other words, reduction of the incubation volume as well as grouping of the embryos can increase blastocyst development in humans [38]. This is most likely the result of the stimulating capacity of embryotrophic ligands such as platelet-activating factor. On the other hand, there is the potential risk of embryo-toxic ammonium accumulation if embryo density is too high. It has been stated that group culture of embryos creates both deleterious and beneficial effects for the embryos, with a net beneficial outcome [39].

1.6. Embryo selection techniques

Irrespective of whether IVF or ICSI was used to generate embryos in culture it is of utmost



Video 1.11 Trophectoderm biopsy (courtesy of Dr Amparo Mercader, IVI Valencia).

importance to effectively select the embryo with the highest implantation potential. This approach would help to limit the number of embryos transferred and as a consequence multiple pregnancy rate, the most severe complication in assisted reproductive technologies, would be significantly reduced.

Naturally, invasive techniques, such as polar body (Video 1.9), embryo (Video 1.10), or trophectoderm biopsy (Video 1.11), most accurately reflect the actual state of health of gametes and embryos, with the latter approach being the most reliable one, since it

maximizes insight while minimizing mechanical harm due to the biopsy process.

Alternatively, the health state of the somatic granulosa cells surrounding the oocyte may be used non-invasively, addressing their mutual dependence [40]. Since both cell types grow in the same surrounding and experience identical conditions in terms of nutrition during folliculogenesis, somatic cells can serve as an indirect marker of the health of the female gamete. With respect to this the rate of apoptosis [41, 42] and the relative telomere length in cumulus cells [43] as well as their transcriptomic profile [44] was successfully used for accurately predicting treatment outcome.

However, selection of the best embryo for transfer in routine IVF/ICSI programs is much rather based on techniques such as morphological analysis. Prediction of implantation can be enhanced if morphological information on gametes, zygotes, and embryos at different stages of pre-implantation development is pooled [45]. Nevertheless, this non-invasive method is far from being a perfect predictor of implantation. Recently, adding time course of mitotic divisions to static morphological observations started the era of morphokinetics, a field that may provide additional help in specifying implantation [46].

1.7. Non-invasive embryo selection based on morphology

Both approaches, morphological evaluation and morphokinetics, focus on proper embryonic development within the first days of in vitro culture. Since not all prospective studies do support prolonged embryo culture up to blastocyst stage it should only be considered in patients with sufficient embryos with good prognosis of blastulation.

Independently of whether transfer is planned at cleavage or blastocyst stage, proper identification of viable concepti is a prerequisite for high rates of implantation, pregnancy, and live birth. Most importantly, repeated screening for morphological features attributed to chromosomal aberrations is strongly recommended from oocyte stage onwards because spontaneous post-zygotic errors do occur and show in vitro persistence throughout pre-implantation development [47]. As a consequence of accurate deselection of oocytes and embryos having a high risk for aneuploidy, euploid concepti will accumulate in culture [45].

It is generally acknowledged that giant oocytes, apart from prophase I eggs showing a germinal vesicle, are the only type of female gamete that are 100% diploid which would cause digynic triploidy in case of ICSI [48]. Although not related to aneuploidy, oocytes with clusters of the smooth endoplasmic reticulum should also be used with caution since cases of stillbirth, malformation, and imprinting disorders have been published [49–51].

At zygote stage, concepti with uneven pronuclear size have to be discarded since the vast majority of them showed aneuploidy [52]. In addition, zygotes with pronuclei located in the periphery of the ooplasm or without abuttal have a bad prognosis in terms of further development [53, 54]. Gianaroli *et al.* [54] reported that the type of pronuclei (e.g., not abutted, unequal size, fragmented), the distribution and size of nucleoli (e.g., small and scattered), and the orientation of polar bodies with respect to pronuclei were highly predictive for the presence of complex chromosomal abnormalities in the developing embryos.

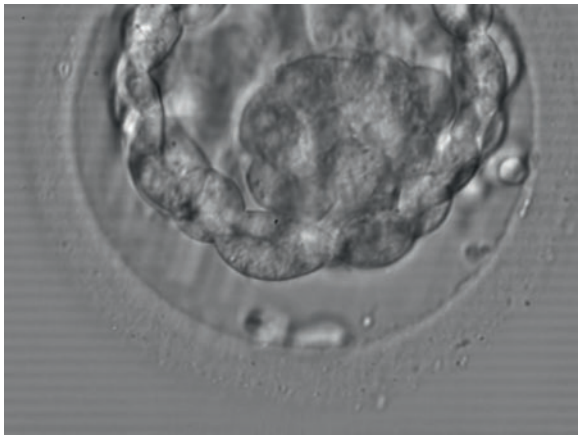
At cleavage stage embryos with bi- or multinucleation and/or uneven blastomeres should be eliminated in order to prevent accidental transfer in case of blastocyst formation [55]. One additional dysmorphism closely related to aneuploidy is the presence of a tetrafoliate-clover shaped arrangement of blastomeres on day 2 [56].

On day 5 of pre-implantation development blastocyst formation is checked and blastocysts are scored according to the quality (based on cell number and cohesion) of inner cell mass and trophectoderm [57].

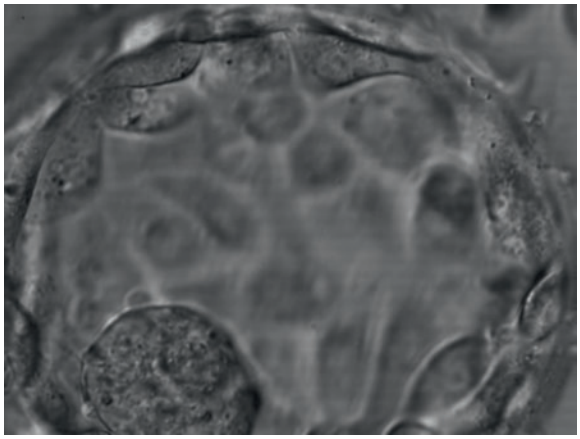
Both cell lineages are not distinguishable at early blastocyst stage but from full blastocyst stage onwards. Although the majority of embryologists tend to choose blastocysts with optimal inner cell mass rather than an optimal trophectoderm, recent papers stress that in terms of live birth actually the latter is of utmost importance [58].

1.8. Embryo transfer

The final step of ART is to transfer one or two thoroughly selected embryos/blastocysts into the uterus approximately 1 cm from the fundus [59]. Embryologists have to carefully load a transfer catheter by aspirating a definite sequence of liquid and/or gaseous phases according to the internal guidelines of their laboratories [60]. It is of importance that in the catheter the culture medium containing the embryos considered for transfer is of minimal volume (e.g.,



Video 1.12 Assisted hatching of an expanded blastocyst (Gardner score 4aa) showing partial collapse.



Video 1.13 Vitrification of a hatching blastocyst (Gardner Score 5aa) using a sequence of media containing ethylene glycol and DMSO (GM501 VitriStore, Gynemed, Lensahn, Germany).

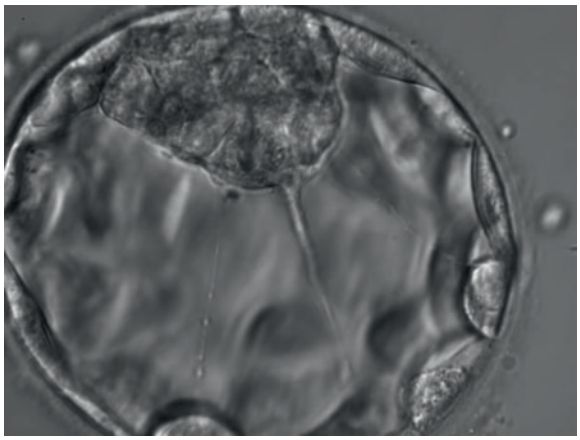
5–10 μl) and the presence of extensive air bubbles is avoided [61].

In order to improve outcome among patients with a history of multiple implantation failures special hyaluronan-enriched transfer media are available [62]. Assisted hatching (Video 1.12) of the candidate embryos in cases of suspicious zona pellucidae, increased female age, or repeated implantation failure is an alternative technique to optimize embryo transfer [63, 64].

Ultrasound guidance allows the clinician to control optimal placement of the embryos [65], a technique which was shown to be of benefit in terms of outcome. After transfer, luteal phase support has been clearly demonstrated to improve pregnancy rates in women undergoing ART. Because of the increased risk of ovarian hyperstimulation syndrome associated with the use of hCG, progesterone has become the treatment of choice for luteal phase support [66].

1.9. Cryopreservation

For ethical reasons surplus zygotes, embryos, and blastocysts of adequate quality are cryopreserved; thus, saving them for usage in later treatment cycles. In principle, two cryopreservation techniques are available: slow freezing and vitrification. Due to a remarkable improvement in the efficiency of vitrification techniques as an alternative to the classical slow-freezing procedure [67], the former approach is



Video 1.14 Artificial shrinkage of the blastocoel by means of an ICSI pipette.

generally accepted as the most efficient technique [68], although all developmental stages, particularly zygotes, can successfully be frozen in a slower mode.

The physical process by which a viscous solution supercools to very low temperatures and finally solidifies into a stable glassy phase, without undergoing crystallization, at a practical cooling rate is called vitrification. According to its definition, with vitrification formation of ice crystals is theoretically impossible both in the intra-cellular as well as the extra-cellular spaces. The fundamental issue in vitrification is to achieve and maintain conditions within

the cells which guarantee an amorphous state throughout the cooling as well as during the warming process. Independent of the carrier device that determines the cooling and/or the warming rate, the key to success in order to achieve a "glass-like" state depends on an optimal balance between the speed of cooling–rewarming (time and temperature) and the optimal cell dehydration and penetration of cryoprotectant when they are exposed to concentrated hypertonic solutions (Video 1.13). Particularly, in blastocysts the amount of water in the blastocoel is highest, bearing the highest risk of cryodamage. In order to avoid this, blastocoel fluid can be removed artificially [69] (Video 1.14), thus increasing survival rate and finally cumulative pregnancy rate.

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