

Kersti Lundin

Unit of Reproductive Medicine,
Sahlgrenska University Hospital, Gothenburg, Sweden

***In vitro* fertilisation – where are we now?**

History

In vitro fertilisation (IVF) – “fertilisation outside the body” – has been a possibility for humans to achieve parenthood since the late 1970s when the first “test tube” baby, Louise Brown, was born. The availability of IVF varies hugely throughout the world, mainly depending on whether there exists a reimbursement system from the National Health Services. For example in Denmark, which has a liberal reimbursement system, almost 5% of all children born are from IVF treatments, while in Latin America the figure is around 0.1%. In Sweden 2006 it comprises 3% of all children born, and it is regarded as an important contribution against the declining population in the western world. In the Nordic countries, 10 to 15% of couples in fertile age are considered to have some sort of fertility problems, making this a large health issue for families.

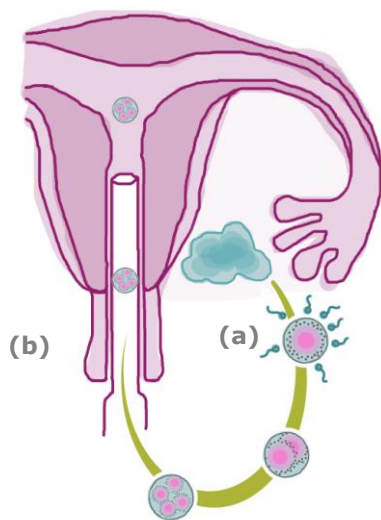


Fig 1. (a) At oocyte pick-up, the oocytes are aspirated using an ultrasound-guided needle passing through the vaginal wall into each follicle of the ovary. (b) At embryo transfer the embryo is loaded into a catheter which is entered into the uterus where the embryo is deposited. (Illustration by Karolina Kristensson.)

IVF started as a treatment for female infertility, mainly tubular damage. Since then the indications have widened, and covers a number of both female and male indications, such as anovulation, endometriosis, hormonal disturbances, vas deferens block, low sperm count.

Hormone stimulation and oocyte pick-up

A monthly ovulation usually results in only one mature oocyte. However, it is rather inefficient – at least using today’s techniques – only to inseminate one oocyte at a time *in vitro*. Therefore, routine methods today involve hormonal stimulation of the woman in order to increase the number of oocytes that mature simultaneously in the ovary, so called controlled ovarian stimulation (COS). By injecting follicle stimulating hormone (FSH) daily for 10-12 days, the hormone levels in the blood and the ovarian tissue are increased, and a higher number of follicles/oocytes are recruited in the ovary. When the follicles have reached a certain diameter (measured by ultrasound), another hormone, human chorionic gonadotropin (HCG), is administered in a single dose. This hormone induces the final stages of follicle development and oocyte maturation. *In vivo*, this final stage is instead started by the hormone luteinising hormone (LH). HCG stimulates the same receptors as LH and thus replaces the natural LH-surge that is trig-

CORRESPONDENCE
to Kersti Lundin
Email: Kersti.Lundin@vqregion.se

gered by rising estradiol levels in the natural hormonal cycle.

Usually between 5-15 mature oocytes can be obtained using this treatment. The oocytes are aspirated from the ovaries by using a transvaginal ultrasound guided needle (Fig 1.) connected to a pump. After this so called "oocyte pick-up" procedure, the oocytes are transferred to culture medium, and kept in a humidified incubator (37 °C, 5% CO₂) until insemination with sperm is performed.

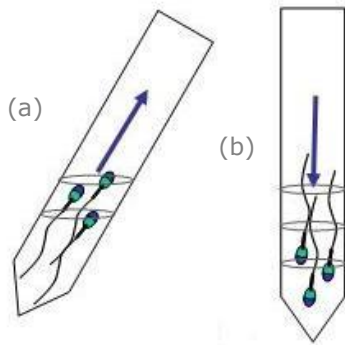


Fig 2. Motile sperm can be separated from the seminal plasma and from immotile sperm and other cells by e.g. the (a) swim-up method where the ejaculate is placed in the bottom of a tube with a layer of buffered culture medium on top. After 45 minutes at 37 °C a large proportion of the motile sperm will have swum upwards into the medium, which is then aspirated and transferred into another tube. In (b) the sperm is centrifuged through gradients of viscous medium. The motile sperm will end up in the bottom of the tube, where they are collected, transferred to another tube, and washed with medium. (Illustration by Kersti Lundin.)

Sperm preparation

While the oocytes are being aspirated, a sperm sample from the man is processed. The goal is to select and concentrate the sperm cells and to separate them from the rest of the ejaculate, called the seminal plasma. The seminal plasma consists of fluid from the testicles, as well as from the prostate and other glands along the male reproductive tract. It supports the sperm with nutritional factors, growth factors and hormones during the passage through the reproductive tract. However, it also contains factors that suppress activation of the sperm and is therefore removed before the oocytes are inseminated with the sperm. This separation can be performed either by letting the sperm swim away from the seminal plasma into a buffered solution ("swim-up"), or by centrifugating the ejaculate onto a layered viscous gradient solution. The live sperm cells will then pass through the layers while the seminal plasma will stay on top (Fig 2).

The last step in the sperm preparation procedure is to dilute the sperm with culture medium into a working concentration, usually around 200.000 sperm per mL. A cell counting chamber is used to count the sperm.

If the man has a very low sperm production, or a normal production but a block in the vas deferens, surgical methods can be used to aspirate sperm directly from the testicle or from the epididymis, where the sperm go through the last stages of maturation (Fig 3).

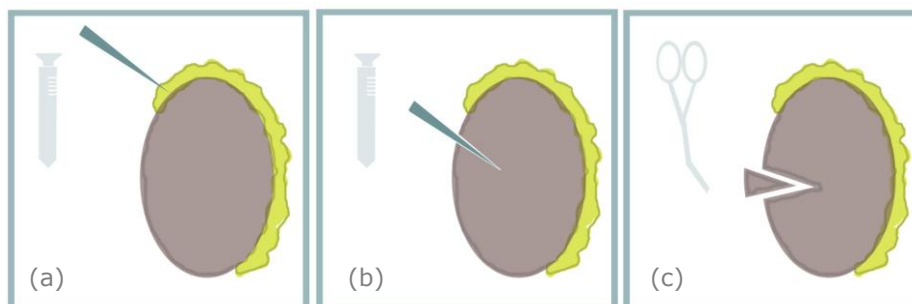


Fig 3. In cases of blocked vas deferens, or extremely low concentrations of sperm in the ejaculate, sperm may be aspirated using a butterfly needle from either (a) the epididymis, or (b) the testis. Sperm may also be obtained by taking a biopsy from the testis (c); however, this is a much more invasive procedure. (Illustration by Karolina Kristensson.)

When only very low numbers of sperm can be collected, microinjection treatment (or ICSI, see below), can be used.

Fertilisation of an oocyte

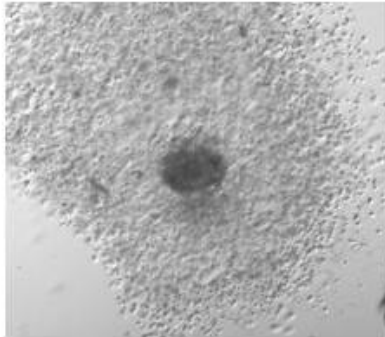


Fig 4. An oocyte surrounded by a large mass of cumulus cells. (Photo by Kersti Lundin.)

When the oocyte leaves the follicle – either by ovulation in a natural cycle or by follicle aspiration in an *in vitro* fertilisation cycle - it is surrounded by several layers of supportive cells, so called cumulus cells (Fig 4). *In vivo*, the fertilisation of an oocyte by a spermatozoon occurs in the fallopian tubes, and involves first penetration of the cumulus cells, then binding to the “zona pellucida” (the eggshell, Fig 5). The sperm then drills its way through the zona pellucida, by using a combination of a propelling movement, and enzymes released from the sperm head. After penetration of the zona pellucida, the sperm travels through the so called perivitelline space between the zona pellucida and the oocyte itself. When it reaches the oolemma (the plasma membrane of the oocyte), the membranes of the sperm and of the oocyte fuse, and the sperm is thereby incorporated into the cytoplasm of the oocyte.

The entrance of more than one sperm into the oocyte is prevented by two mechanisms. One is the so called fast vitelline block, where the first sperm that reaches the oolemma induces an immediate (milliseconds) sharp increase in the oolemma membrane potential, preventing more sperm from attaching and fusing to the oolemma. The second, slower but more longlasting mechanism, is a release of so called cortical granules from the oolemma. These granules contain enzymes that cause the zona pellucida to harden and the sperm binding ligands of the zona to break down, thus preventing further sperm from binding to or penetrating the zona pellucida.

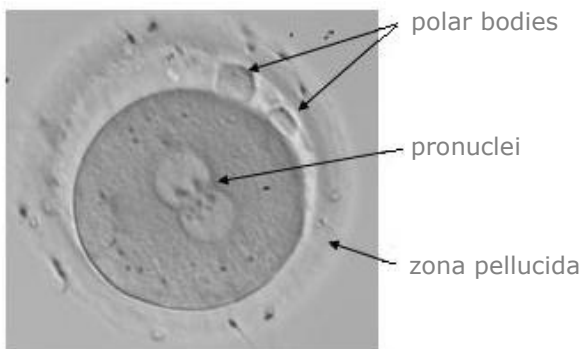


Fig 5. A fertilised oocyte (zygote) showing two pronuclei, one from the sperm and one from the oocyte. The pronuclei will fuse in a couple of hours to form the new diploid genome. The two polar bodies (containing DNA extruded from the oocyte during meiosis I and meiosis II) can be seen in the perivitelline space between the zona pellucida and the oocyte membrane (oolemma). (Photo by Kersti Lundin.)

Once inside the oocyte, the sperm DNA forms a sperm (male) pronucleus. This pronucleus contain half the male DNA, i.e. one single copy of each chromosome. Factors released from the sperm cytoplasm activate the oocyte via calcium fluxes to form the oocyte (female) pronucleus, consisting of one single copy of each of the female chromosomes (Fig 5). The oocyte is now fertilised. After about 5-10 hours the two pronuclei will fuse, and the new diploid genome, with half of the chromosomes from the sperm and half from the oocyte, is thereby formed.

Methods of *in vitro* fertilisation

At present, two methods are used to fertilise oocytes *in vitro*. The original way is to bring together oocytes and processed (separated from the seminal plasma) sperm in culture medium in a petri dish and leave it overnight in an incubator. This procedure mimics the *in vivo* situation, i.e. the sperm has to be able to penetrate the cumulus cells, the zona pellucida, and to fuse with the plasma membrane of the oocyte.

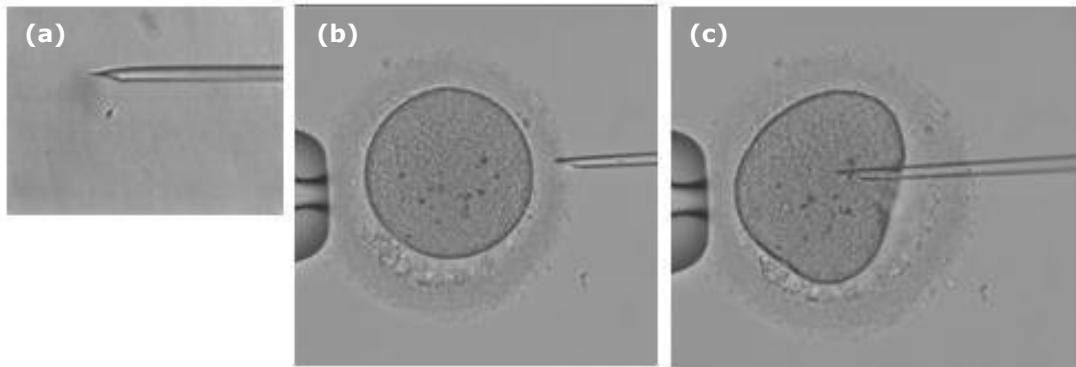


Fig 6. During the ICSI procedure the sperm is aspirated into the injection pipette (a). The oocyte is held by suction to a so called holding pipette (b) and the injection pipette containing the sperm is positioned onto the zona pellucida. The injection pipette is passed through the zona pellucida (c) and into the cytoplasm of the oocyte (ooplasm) where the sperm is deposited. (Photos by Kersti Lundin.)

A more novel method is to inject a single sperm into the oocyte (microinjection or ICSI, intracytoplasmic sperm injection). ICSI is used when only a low number of sperms are obtained, or when a previous "routine" IVF cycle has failed to fertilise. One single sperm is aspirated into a very thin needle which is connected to a hydraulic micromanipulator system and an inverted microscope. The needle containing the sperm is inserted through the zona pellucida and through the ooplasm, and the sperm cell is deposited into the oocytes' cytoplasm (Fig 6). Before performing ICSI the oocyte is "denuded", i.e. the cumulus cells are removed by a combination of enzymatic treatment and mechanical "scrubbing" (the oocyte is aspirated in and out of a thin pipette, compare Fig. 4 and 5). During the ICSI procedure, the oocytes are kept in a petri dish in 10-15 µl droplets of buffered medium covered with warm mineral oil (Fig 7).

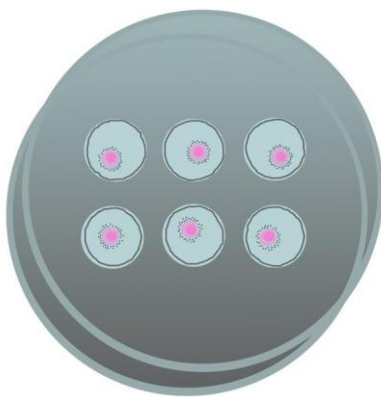


Fig 7. The oocytes and embryos can be cultured either together in an open system (0.5 mL medium without oil cover, not shown) or individually in a petri dish with droplets made out of culture medium and covered with mineral oil. (Illustration by Karolina Kristensson.)

When performing ICSI, the sperm does not have to be able to swim, or to penetrate the zona pellucida or the plasma membrane. However, in order to be able to fertilise the oocyte, it has to have the ability to decondense its nuclear material (forming a male pronucleus), and to activate the oocyte to go through the second meiosis, to form the female pronucleus.

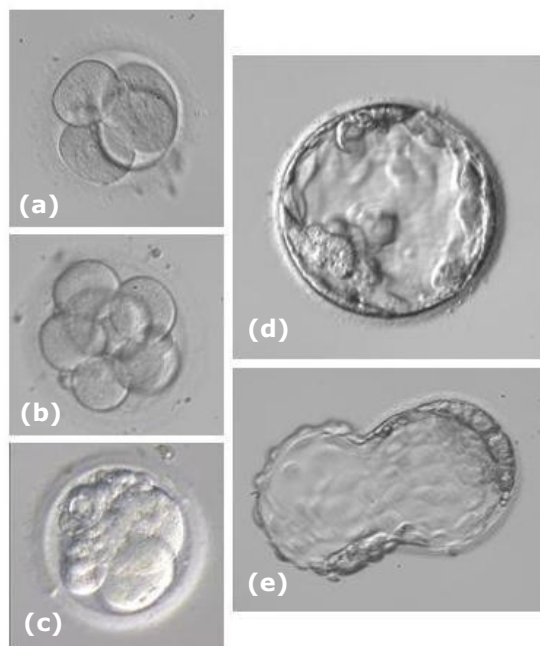
Embryo culture and development, embryo transfer

After insemination or microinjection, the oocytes are cultured in petri dishes, either in an open system in approximately 0.5 mL of culture medium, or in small droplets under mineral oil. In order to keep the temperature, pH and osmolarity stable, the dishes containing the oocytes/embryos are kept in warmed incubators with specified gas mixtures and high humidity (Fig 7).

Sixteen to 18 hours after insemination/injection, the oocytes are checked for fertilisation. A normally fertilised oocyte (called a zygote) has two pronuclei (one from the sperm and one from the oocyte) and two so called polar bodies that contain the DNA extruded from the oocyte while going through meiosis I and II (Fig 5).

To obtain acceptable pregnancy rates, embryo selection is very important. The main parameters scored at present are cleavage speed and embryo morphology. An embryo should cleave at an optimal rate, not too slow and not too fast. It has been shown that an embryo with 4 cells, day 2 (44 hours after insemination) and 8 cells, day 3 (68 hours after insemination) has a higher implantation rate than both slower and faster cleaving embryos. A "good quality embryo" should also have no or little cell fragmentation, have cells of equal size, and never have more than one visible nucleus in each cell (Fig 8). An embryo that has been cultured to the blastocyst stage (5-6 days after insemination), is comprised of approximately 100-200 cells. A "good quality blastocyst" should be expanding (growing in diameter due to a fluid-filled cavity), have distinct inner cell mass and trophectoderm layers, with no degenerative cells (Fig 8).

Fig 8. (a) A "top quality" embryo two days after fertilisation, with four even-sized cells and no fragmentation. (b) A "top quality" embryo at day three, with eight even-sized cells without fragmentation. (c) A poor quality embryo at day two, with three uneven-sized cells and considerable amounts of fragments. (d) A top quality blastocyst at day 5, showing expansion (enlargement), clear distinctive inner cell mass and trophectoderm cells. (e) A blastocyst hatching out from its zona pellucid (seen here as a thin shell covering the right half) on day 6. (Photos by Kersti Lundin.)



Transfer of the embryo to the uterine cavity can be performed on day 2-3 post insemination/injection (4-8 cells, "cleavage stage") or at day 5-6 (100-200 cells, blastocyst stage). The embryo(s) is aspirated into a transfer catheter together with a small amount of culture medium. Guided by ultrasound, the catheter is inserted via the vagina into the uterus, where the embryo is deposited (Fig 1).

Pregnancy and implantation

If no bleeding has occurred 2-3 weeks after the embryo transfer (ET), a pregnancy test can be made. If this is positive, approximately 6-7 weeks after ET an ultrasound is made in order to establish the existence of a fetal sac, foetus and beating heart.

Pregnancy rates can be calculated either per oocyte pick-up, or per embryo transfer. It can be presented as pregnancies in total (= a positive HCG test) or as only clinical pregnancies (= confirmed by ultrasound). Pregnancies that cannot be confirmed by ultrasound (due to a very early spontaneous abortion) are then counted as biochemical pregnancies. The most important outcome is of course the birth of a healthy baby, something which occurs in approximately 20-40% of every started hormone stimulated cycle.

"Implantation rate" is defined as number of foetuses per number of transferred embryos, i.e. it is a measure of how many of the embryos that are transferred back to the women that are able to implant and develop into a foetus.

As a result of improved knowledge and technology in *in vitro* fertilisation treatments, pregnancy and implantation rates have increased. This has resulted in increased numbers of multiple pregnancies and multiple births. In some countries, high incidences of both triplets and quadruplets are recorded. This has led to demands from health services and society, and from the profession itself, of a reduction of the number of embryos that are transferred to the woman. In Sweden, recommendations from the National Board of Health and Welfare from 2003 state that only as an exception should more than one embryo be transferred. The results of this is that from 2003 to 2006, in Sweden, the percentage of patients receiving only one embryo has increased from a mean of less than 20% to around 70%. During the same time, the percentage of twins has been reduced from almost 30% to less than 10%.

Many countries in the Northern Europe are now adopting similar strategies, while large parts of the world are still discussing a reduction to two, or even to three, embryos for transfer.

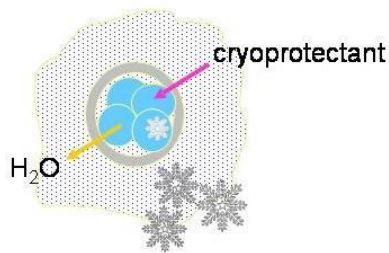


Fig 9. During the freezing of an embryo, increasing concentrations of cryoprotectant outside the cells will draw intracellular water out of the cells by osmosis, and cryoprotectant into the cells. At thawing, the embryo is placed into a medium with low concentration of cryoprotectant (lower than in the cells), which will reverse the process. (Illustration by Kersti Lundin.)

Fig 10. (a) Showing a straw and an ampoule, that can be used for cryopreservation of embryos and/or sperm. (b) Cryopreserved cells are kept in holders in large tanks filled with liquid nitrogen. (Photos by Kersti Lundin.)

Cryopreservation

“Good quality” embryos, i.e. embryos that are believed to have a high potential for implantation, and that are not used for immediate (“fresh”) transfer, may be cryopreserved and replaced in a later hormone cycle. During freezing, the embryos are passed through increasing concentrations of a cryoprotectant (usually propanediol or ethylene glycol) to replace the intracellular water, in order to minimise the creation of ice crystals during the process (Fig 9). Cryopreserved embryos can either be kept in ampoules, or more commonly in small sealed straws kept in liquid nitrogen tanks (-196 °C) (Fig 10), where they may be stored for years. Different countries have different legislation and/or recommendations for how long embryos may be kept cryopreserved, usually between 1-5 years. In some countries e.g. Italy and Germany, cryopreservation of cleaved embryos is not allowed. However, they may be preserved as either unfertilised oocytes, or as fertilised, non-cleaved embryos (“zygotes”).



Thawing of embryos is performed “reversed” to freezing, i.e. they are passed through a series of more and more diluted cryoprotectants, and the cryoprotectant inside the cells is by the osmotic process again replaced by water.

Cryopreservation of embryos is today a completely integrated part of the IVF treatment and a large contribution to the cumulative pregnancy rates. Especially in countries where single embryo transfer is becoming the most common routine, cryopreservation of excess good quality embryos is becoming increasingly efficient and important. Large studies have shown that replacement of cryopreserved embryos may increase the cumulative pregnancy rate for an IVF patient by 15-20%.

Results

Big efforts are being made to collect IVF data from centres around the world. In Europe, ESHRE (European Society for Human Reproduction and Embryology) collects data from a majority of centres, the same is being done in USA by SART (Society for Assisted Reproductive Technology). These huge sets of compiled data contain numbers of cycles (fresh and frozen-thawed), numbers of embryos transferred, pregnancy data etc. split up per a large number of variables. In the last couple of years, also data concerning e.g. outcome for IVF children and success rates for preimplantation genetic diagnosis are being collected.

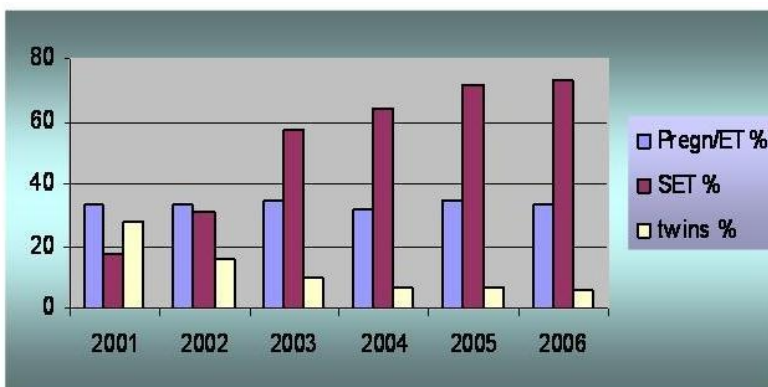
Due to the time it takes to collect and analyse the data, the presentation lags some years behind, and the data for Europe 2002 were thus published in 2006. From that paper, where data from 25 countries and 631 clinics are presented, it can be seen that 257 682 IVF cycles and 57 162 frozen-thawed embryo transfers were performed. The overall clinical pregnancy rate per transfer was 29.5% and the multiple birth rate was 24.5%.

The differences between countries are very large, and in a comparison it can be shown that the clinical pregnancy rate per transfer and the multiple birth during the same period (2002) for Sweden was 34.0% and 19.4%. In Fig. 11 the results from Sahlgrenska University Hospital, Gothenburg, Sweden 2001-2006 are presented.

Future possibilities

Since the start of IVF, success rates have increased continuously. Although it may look as if the success rates have not increased in Sweden for the last years (Fig 11), it has to be taken into consideration that the trend of replacing fewer embryos lowers the pregnancy

Fig 11. Data from Sahlgrenska University Hospital 2001-2006. The pregnancy rate per transfer (Pregn/ET) has been kept constant despite that the proportion of single embryo transfers (SET) has increased from less than 20% to over 70%, and the mean number of embryos for transfer has been reduced from 1.9 in 2001 to 1.2 in 2006. Twin births have at the same time been reduced from almost 30% to 6%. These figures can be considered to be fairly representative for Sweden. (Illustration by Kersti Lundin.)



rates compared with when a higher number of embryos are replaced. Still, despite replacing fewer embryos the

pregnancy rates have not decreased, but remained stable (Fig 11). This can be explained by an increased implantation rate. An increased implantation rate means that a higher proportion of the replaced embryos actually implant into the uterus. The reasons for this may be several; e.g. better hormone stimulation protocols, improved *in vitro* culture conditions and improved embryo selection methods.

Approximately 60% of all couples that come to a clinic in Sweden due to fertility problems, will end up with the birth of a child after treatment, after a mean of 2.3 embryo transfers (using fresh or frozen-thawed embryos).

However, there is presumably still room for improvement. For example, it is known that also morphologically "good" embryos have a high rate of chromosomal and probably also metabolic errors. Thus, we need methods that will enable us to find not only the morphologically most optimal embryos, but among those the ones without chromosomal errors and with an optimal metabolism.

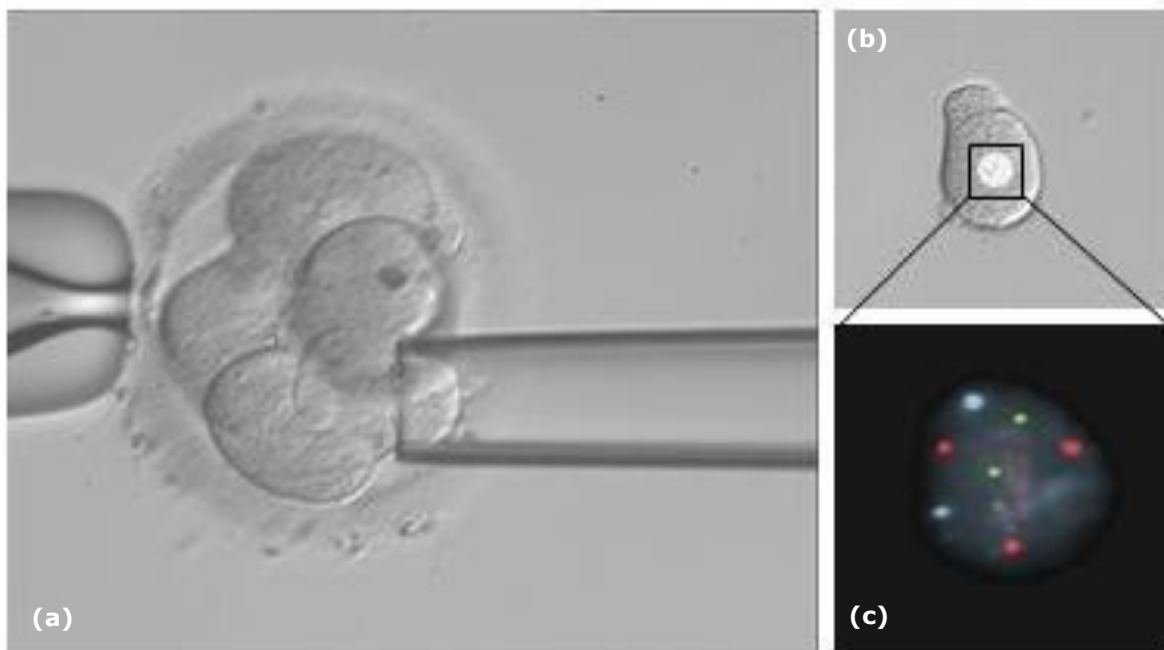


Fig 12. During preimplantation analysis, one or two cells are biopsied from the embryo (a). The cell(s) is fixed onto a glass slide (b), and the cytoplasm is removed so that only the nucleus remains. The nucleus is hybridised with fluorescent probes for selected chromosomes (c). (Photos: (a) and (b) by Kersti Lundin; (c) by Charles Hanson)

One method that is being used, although it is still controversial, is the use of preimplantation genetic screening (PGS). Originally, this technique was developed in order to find non-affected embryos for couples with a severe hereditary disease (then called preimplantation genetic *diagnosis*, PGD). However, in the last couple of years the same method has also been used in order to try and find the chromosomally most optimal embryo for couples in ordinary IVF treatments. In PGS, one or

two cell(s) is biopsied out from the embryo on day 3 after fertilisation (= 8 cell stage) (Fig 12). This cell is analysed by fluorescence in situ hybridisation (FISH) for chromosomal errors, and a chromosomally normal embryo may thereby be selected and transferred. For couples with severe hereditary diseases the PGD method is important and has relatively good results, but PGS does not seem to work quite as well when it is used in the aim of enhancing embryo selection and birth rates. There could be several reasons for this; the biopsy procedure may be detrimental to the embryo development, or, choosing a chromosomally normal embryo instead of the morphologically "most good-looking" embryo may not be optimal. We also know that human embryos at the eight cell stage have a rather high degree of mosaicism, and the cell that has been removed for biopsy may not be representative for the whole embryo. In addition, it is at present not known how much of chromosomal errors an embryo can have and still survive and develop. If only one or a few cells out of the eight are abnormal, the embryo might perhaps still be able to develop and implant, in which case the pre-implantation analysis would not have an impact.

The ultimate embryo selection method would be if we could just take a small sample from the medium in which the embryo has been cultured, analyse it, and find the "top" embryo, i.e. the one with the highest potential to develop and to implant in the uterus. Some studies today suggest that this may be possible in the not too distant future. Possible metabolic markers could be amino-acid turn-over, and/or HCG metabolites. Also micro-array analyses of one cell of the embryo has been put forward as a possible method for selecting the metabolically fit embryos.

In conclusion, *in vitro* fertilisation can today be considered as a more or less routine and rather straightforward treatment for many infertility/subfertility indications. The results are quite good, approximately 60% of all couples with a fertility problem will have a child after 1-3 oocyte pick-ups, including the following frozen-thawed transfers. The risks involved with assisted reproduction are considered to be small. The high rate of multiple pregnancies and births has been considered the largest risk to mother and child. However, the decrease of numbers of embryos for transfer is now beginning to reduce the frequency of multiple pregnancies in many countries.

Exercises

With help of the following link;

http://www.eshre.com/file.asp?filetype=doc/04/008/eim_2002.pdf

find out:

1. Which European country had most IVF-treatments during 2002?
2. Which European country had most twin births in connection with IVF-treatment?

Reference literature/links

Oxford Journals; Human Reproduction

http://humrep.oxfordjournals.org/misc/free_articles.dtl

A textbook of in vitro fertilization and assisted reproduction. The Bourn Hall guide to clinical and laboratory practice (1999). (Second edition) ed. Brinsden, PR. London: Parthenon Publishing. ISBN: 1-85070-000-1

Laboratory aspects of in-vitro fertilization (2000) (Second edition) eds. Elder, K and Dale, B. Cambridge: Cambridge University Press. ISBN: 0 521 77863 8

Infertilitet (2005) eds. Hreinsson J, Hamberger L and Hardarson Th. Lund: Studentlitteratur. ISBN: 91-44-03867-4

Nyboe Andersen A., Gianaroli L., Felberbaum R., de Mouzon J. and Nygren K.G. (2006) Assisted reproductive technology in Europe, 2002. Results generated from European registers by ESHRE. *Human Reproduction* 21 (7)1680-1697

Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine. (2007) Assisted reproductive technology in the United States: 2001 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology registry. *Fertility and Sterility* Feb 1; [Epub ahead of print]