Laboratory aspects of assisted reproduction

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A number of advances have been made concerning the laboratory aspects of assisted reproduction. Intracytoplasmic sperm injection has revolutionised the treatment of male infertility. With the development of better embryo culture media, blastocyst transfer is now possible and is likely to reduce high-order multiple pregnancy in assisted reproduction treatment. Pre-implantation genetic diagnosis has become an alternative to prenatal diagnosis. The recent use of molecular biology techniques to detect small genetic defects in men with severe male-factor infertility has provided information for the better counselling of these patients. Other techniques that are being developed are likely to have a tremendous impact on assisted reproduction treatment. These include in vitro maturation, follicle culture, and oocyte/ovarian tissue cryopreservation. The current status of the developments in the laboratory aspects of assisted reproduction is reviewed in this article.

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Introduction

Assisted reproductive technology has revolutionised the treatment of infertility. In this paper, the major recent advances in the laboratory aspects of assisted reproduction will be reviewed in two parts. The first part will review the relatively recently developed techniques that have become widely used in assisted reproduction. The second part will focus on techniques that are currently being developed and are likely to have a significant impact on assisted reproductive technology.

Established techniques

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) has had a considerable impact on the treatment of male-factor infertility. The technique involves the injection of a single spermatozoon into an oocyte. Since its development in 1992,[1] ICSI has helped subfertile couples at most assisted reproduction units. The fertilisation outcome after ICSI is little affected by most conventional criteria of sperm quality. Since the requirement for spermatozoa during ICSI is minimal, ICSI can be used in almost all men in whom spermatozoa can be found in the ejaculate, epididymis, or testis.

The first baby conceived using ICSI in Hong Kong was born in 1995 at the Queen Mary Hospital (QMH). In 1998, the fertilisation rate of the ICSI technique at the QMH was approximately 60%.[2] The results of ICSI with spermatozoa obtained from the ejaculate, by microsurgical epididymal sperm aspiration, or by testicular sperm extraction are shown in Table 1.[2]

Studies of obstetric outcomes, the malformation rates, and karyotype abnormalities of children born after ICSI have been reported by a number of groups.[3]

Table 1. Outcomes of intracytoplasmic sperm injection at the Queen Mary Hospital (1998)[2]

	ICSI* alone	MESA† + ICSI	TESE‡ + ICSI
Fertilisation rate	64.0%	62.4%	55.4%
Mean No. of embryos per transfer	2.5	2.2	2.2
Pregnancy rate per transfer	21.5%	20.8%	18.2%
Multiple pregnancy rate	30.4%	40.0%	-

* ICSI intracytoplasmic sperm injection
† MESA microsurgical epididymal sperm aspiration
‡ TESE testicular sperm extraction

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In general, these children are not associated with a higher incidence of congenital malformations, compared with those derived from conventional in vitro fertilisation (IVF). However, a slight increase in the incidence of chromosomal abnormalities has been shown in a follow-up study of ICSI children.[4]

In a recent study that used sibling oocytes from couples with tubal infertility and normospermic semen, no difference was seen in the implantation potential of embryos obtained by conventional IVF and ICSI.[5] However, the use of ICSI for all couples requiring assisted reproduction should be discouraged for two reasons. Firstly, male infertility has been associated with a number of genetic defects, such as Y-chromosome microdeletion and chromosomal aneuploidy. The chance of having such an abnormality is higher in patients with severe male-factor infertility.[6,7] The transmission of genetic defects from the father to the ICSI-derived baby has been demonstrated by a study in Hong Kong[8] and elsewhere.[9] Abnormal spermatozoa, which would normally have a low or no chance of fertilising an oocyte, are able to do so with ICSI because there is no means of distinguishing them from the normal spermatozoa at the time of ICSI. Thus, the indiscriminate use of ICSI propagates genetic defects in abnormal spermatozoa. Secondly, transmission of foreign DNA by ICSI has recently been demonstrated.[10] This has raised concerns that ICSI might transfer infectious materials to the next generation.[11]

When no spermatozoa can be found, spermatid injection has been suggested as an alternative, and pregnancies have been obtained with this procedure.[12] The outcome, however, is much less satisfactory than when ICSI with mature spermatozoa is used.[13] There are two inherent technical problems with this procedure. Firstly, it is difficult to identify round spermatids among all the other round cells in the testicular biopsy. Secondly, round spermatids from men with complete spermiogenesis failure are more likely to have apoptosis-related DNA fragmentation than are spermatids from men in whom late elongated spermatids or spermatozoa can occasionally be found.[14] These problems can be partially solved by culturing the germ cells in vitro. This procedure yields spermatids that have a higher degree of cytoplasmic maturation, thereby facilitating spermatid recognition.[15] This technique also helps to overcome the danger of inadvertently using apoptotic spermatids for ICSI.[16] A pregnancy from the injection of spermatid that matured in vitro has recently been reported.[17] The injection of round spermatids was rendered unnecessary by Silber et al,[18] who found that the presence of round spermatids was always associated with the presence of elongated spermatids or mature spermatozoa in the testicular biopsy.

Pre-implantation genetic diagnosis

The development of molecular biology techniques has made it possible to identify chromosomal and gene defects in just one or two cells. This advancement has led to the development of pre-implantation genetic diagnosis (PGD),[19] which can detect many common chromosomal abnormalities and inherited single-gene defects. In this procedure, one or two blastomeres, usually from an eight-cell–stage embryo, are removed and the genetic make-up in the extracted blastomeres is determined. The fundamental assumption is that these blastomeres are identical to those in the rest of the embryo. Hence, only embryos from which extracted blastomeres show normal genomes are transferred. Similarly, the polar body of oocytes or zygotes can be used for PGD.

There are two methods for detecting genetic abnormalities in a single cell. Polymerase chain reaction (PCR) analysis is used to detect genetic abnormalities at gene level. Although this technique is very sensitive, it does not provide information about the chromosome copy number. In contrast, multicolour fluorescent in situ hybridisation (FISH) not only detects abnormalities in autosomes, but also the number of sex chromosomes.[20] Currently, FISH is the preferred method of identifying the sex of an embryo and of detecting aneuploidy when women are of advanced maternal age. The detection of aneuploidy by PGD in human embryos has recently been shown to reduce embryo loss after implantation, although the implantation rates were not significantly improved.[21]

Pre-implantation genetic diagnosis may not be absolutely accurate, because of the occurrence of chromosomal mosaicism in human embryos—that is, two or more blastomeres from the same embryo having chromosome complements that are different from the remaining blastomeres. Studies using FISH have shown that a significant number of normally developing embryos display chromosome mosaicism.[22,23] These chromosomal abnormalities are related to the impairment of embryo development, advanced maternal age, embryo culture conditions, and hormonal stimulation.[24] Another reason that PGD may not be absolutely accurate is the failure of PCR amplification of one of the gene alleles, usually termed as allelic dropout. To minimise this effect, PCR conditions need to be optimised for each specific gene allele prior to PGD.

It is now possible to screen for single-gene disorders and chromosomal abnormalities simultaneously in a single blastomere.[25] Attempts have also been made to develop a simple method of karyotyping metaphase chromosomes of the human blastomere.[26]

Co-culture versus sequential culture

It is generally accepted that the culture conditions for human embryos are suboptimal. Two methods have been used to solve this problem: co-culture of embryos with somatic cells and the use of different culture media for different stages of embryo development (sequential culture). Co-culture received considerable interest in the early 1990s, with various studies having shown that co-culture improves the morphological development and implantation rate of treated embryos. Co-culture is particularly helpful to infertile couples with recurrent implantation failure. The oviductal cell is the most logical somatic cell to be used for co-culture with early pre-implantation embryos, as the oviduct is the natural site in which these embryos grow during early development. We have previously shown that co-culture with human oviductal cells improves the maturation rate of blastocysts and decreases the fragmentation rate of developing human embryos, and that improvement is more marked when co-culture is started earlier.[27] In a randomised controlled trial, co-culture with human oviductal cells also enhanced the implantation rate of human embryos in the assisted reproduction programme at the QMH.[28] However, co-culture involves complicated laboratory techniques and screening, and hence it is not suitable for routine use.

Cleaving embryos normally develop in the oviduct, whereas the morulae and blastocysts develop in the uterine cavity. These two stages of embryo growth have different nutritive requirements, which match quite well with the constituents in the oviduct and the uterine cavity. Thus, it is not surprising that the use of sequential culture media specific for different stages of embryo development produces better blastocysts in vitro; the resultant pregnancy rate is at least as good as that seen with co-culture.[29] Because of its simplicity, sequential culture is rapidly becoming the method of choice for producing human blastocysts.

Blastocyst transfer

Early-embryo culture media do not support the long-term growth of human embryos. Hence, assisted reproduction programmes usually involve the transfer of day-2 or day-3 cleaving embryos to the uterus—an unusual site for early embryo development. Co-culture and sequential culture allow the production of sufficient blastocysts to make blastocyst transfer practical. There are a number of advantages of blastocyst transfer. Firstly, blastocysts are transferred to a natural site of development so the development of embryos and the endometrium are better synchronised. This synchronisation may theoretically enhance later implantation. Secondly, in some women, the uterus may fail to give adequate support to cleaving embryos, thereby resulting in the failure of IVF. Blastocyst transfer may overcome this problem. Blastocyst culture may be useful in patients with multiple implantation failure,[30] although these data await confirmation by randomised studies. Thirdly, blastocyst transfer allows better selection of embryos; only good-quality embryos are able to develop into blastocysts. As a result, fewer embryos can be transferred. High-order multiple pregnancy can be eliminated by the transfer of just two embryos.[31] Fourthly, the risk of miscarriage may be reduced, because chromosomally abnormal embryos tend to have retarded development and are less likely to be selected for transfer.[32]

A problem with blastocyst transfer is that only approximately half of the fertilised eggs develop into blastocysts.[29,33] Some patients may therefore have no embryos available for transfer. By carefully selecting patients, the percentage of patients without blastocysts could be low.[34] Although the failure of blastocyst formation in vitro may indicate poor quality of the oocyte, sperm, or embryo,[35] it is also possible that the current sequential media are still not yet optimal. Some embryos that fail to reach the blastocyst stage in vitro might have given rise to offspring, had they been transferred earlier. Studies of methods that further improve currently used sequential culture system media are ongoing. At the QMH, a human oviductal cell–mouse embryo co-culture system has been developed for this purpose. Our data show that human oviductal cells improve the development of mouse embryos in vitro by the production of at least three embryotrophic factors.[36,37] In future, the supplementation of these factors to the culture medium may further enhance human embryo development.

With the increasing use of blastocyst culture and transfer as a routine treatment in human clinical in vitro fertilisation, there is an increasing need for a reliable procedure to cryopreserve blastocysts. The conventional slow-freezing protocol is suboptimal for blastocysts, as the implantation rate of frozen blastocysts is only approximately half of that reported for the transfer of fresh blastocysts.[38] A new vitrification method is being developed, with promising results.[39]

Blastocyst transfer versus cleaving embryo transfer

Although blastocyst transfer has provided good results, Edwards and Beard[40] have questioned whether

blastocyst culture is necessary in selecting embryos with good implantation potential. Scott and Smith[41] were able to select pronuclear embryos with an implantation rate of 28%. Similarly, Van Royen et al[42] have demonstrated that the transfer of a single day-3 embryo could have an acceptable pregnancy rate if a top-quality embryo were available. The implantation rates of the selected embryos in their study were 35% and 49% with one or two top-quality embryo transfers, respectively. These implantation rates are similar to those achieved by the transfer of blastocysts, as reported by Gardner et al.[29] The approach of transferring early embryos using new criteria for good-quality early embryos is attractive, because it involves a shorter culture time at a lower cost, and avoids the risk of having no blastocyst available for transfer.

There is, however, concern about the possibility of selecting good early-cleaving embryos before the activation of the embryonic genome, which occurs at day 3 in human embryos. In an elegant analysis of published reports, Edwards and Beard[43] hypothesised that cell determination in embryos might occur very early in embryonic development, before the supposed time for embryonic genome activation. This cell determination is manifested morphologically as polarity of the oocyte/embryo. In line with their proposal are the findings that the transcription levels at the one-cell stage are critical for later development.[44,45] Recent studies have also shown the occurrence of polarity in early human embryos.[46,47] Thus, important information may be obtained from very early embryos that allow the selection of those with the best implantation potential.

Managing male genetic abnormalities

Recent studies have shown that men with severe male-factor infertility have a higher incidence of chromosomal or genetic abnormalities. Attention has been made to microdeletions in the Y chromosome of these men. The *DAZ* (deleted in azoospermia) gene is often found to be deleted in these men. Preliminary data from the QMH have shown that 9.1% of men with the non-obstructive azoospermia or severe oligospermia in the Chinese population in Hong Kong have *DAZ* deletions.[7] Transmission of a *DAZ* deletion from the father to his ICSI-derived son has also been demonstrated at the QMH.[8]

Developing techniques

In vitro oocyte maturation

While the use of ICSI overcomes the fertilisation problems associated with culturing immature oocytes, there is an increased interest in retrieving oocytes without gonadotrophic stimulation or with limited gonadotrophic stimulation and then maturing them in vitro for embryo transfer. The advantages of maturing oocytes in vitro are that only a minimal amount of expensive drugs are needed; there is no risk of ovarian hyperstimulation, deep vein thrombosis, or other possible long-term side effects of fertility drugs including ovarian cancer; and that a large pool of pre-antral and antral oocytes is theoretically available in the early follicular phase of the cycle. Human oocytes can be matured from small antral follicles, fertilised in vitro, and give rise to live births following embryo transfer.[48,49] Currently, the success rate with in vitro oocyte maturation is low and the culture system needs to be improved.

Oocyte cryopreservation

The ability to freeze and store human oocytes successfully allows the circumvention of moral, ethical, and legal problems associated with embryo freezing. Oocyte cryopreservation also preserves the reproductive potential of women who, for a variety of medical reasons, are likely to lose ovarian function prematurely. Banks of frozen donated oocytes would facilitate the donation process by waiving the requirement for donor-recipient synchrony.

Gook et al[50] have demonstrated that oocytes can survive the conventional embryo cryopreservation protocol without compromising spindle integrity, and with normal karyotype.[51] Pregnancies and live births of cryopreserved oocytes that have been fertilised with ICSI have been obtained.[52] However, the success rate of oocyte cryopreservation remains low and new methods for human oocyte cryopreservation are being investigated.[53]

Cryopreservation of the ovarian cortex

An alternative to cryopreserving mature human oocytes is to cryopreserve ovarian tissue that contains immature oocytes within the ovarian cortex. This approach has the additional advantage over oocyte cryopreservation of preserving the fertility of cancer patients. Detecting the cancer early and immediately starting treatment with chemotherapy and/or radiotherapy improves the long-term survival for patients with many types of cancer.[54] Unfortunately, these treatments may render women either temporarily or permanently infertile and the urgency of such treatments precludes harvesting mature oocytes from many of these patients. Cryopreserving the ovarian cortex would be a suitable option in these cases. The transplantation of cryopreserved ovarian tissue has been able to restore fertility in animals, including marmosets.[55-57] However, efficacy of this technique in humans has yet to be determined.

Follicle culture

In biopsy samples of ovarian cortex, the numbers of primordial follicles present are significantly higher than those in the later development stages.[58] Cryopreserved ovarian tissue of cancer patients can be used for transplantation, but this would carry a risk of transmission of the malignancy.[59] The other option is to culture the primordial and primary follicles that have come from the cryopreserved biopsy sample of cortical tissue. In studies of mice, live offspring have been born from oocytes that have matured from primordial follicles in vitro.[60] Although it is possible to isolate primordial and primary human follicles, it has not been possible to establish long-term culture of these follicles.[61] The development of human follicles in vitro is better in tissue slices than in the partially isolated form.[62] In ovarian slices, human primordial and primary follicles can be cultured to reach secondary and occasionally early-antral follicles.[63]

Conclusion

A number of advances have been made in the laboratory aspects of assisted reproduction in the past decade. These improvements have extended the range of patients to be treated by assisted reproduction. With the use of molecular biology, more is known about the genetic causes of infertility, so better counselling can be offered to the infertile patients. Although a better understanding of the development of the oocyte/follicle and embryo has allowed us to provide a better culture environment for the oocyte and embryo, further research is required to find the ideal culture media.

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