Endocrine, *vol. 9, no. 1, 15–25, August 1998* 0969–711X/98/9:15–25/$10.75

Review

Recent Advances in Assisted Reproductive Technologies

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Assisted reproductive technologies (ART) have received considerable attention, both clinically and empirically. Drs. Steptoe and Edwards removed one oocyte surgically from a woman with infertility related to tubal disease. They fertilized this oocyte in vitro and transferred the formed embryo to the woman's uterus and achieved pregnancy and delivery. The technique of in vitro fertilization (IVF) and embryo transfer (ET) quickly became widely utilized for other causes of infertility as well as for tubal disease. In the last 5 years there has been a number of new developments that are reviewed in this article. The most important and now widely practiced technology has been direct intracytoplasmic injection (ICSI) of the husband's sperm into the wife's oocyte. This was developed for treatment of infertility related to low sperm count. Subsequently it was shown that sperm can be aspirated from epididymis or found in testicular biopsy in obstructive azoospermia. Another promising development is in vitro maturation (IVM) of immature oocytes. This has the potential of avoiding ovarian hyperstimulation, which can be uncomfortable and occasionally dangerous. Some oocytes are unable to fertilize and/or develop into normal embryos. It may be possible that the problem is with the machinery of cytoplasm of the oocyte. Therefore cytoplasmic transfer from a normal oocyte to an abnormal oocyte may overcome the problem. Infertile couples may be faced with many psychological problems that become even more complex with various treatments. Whereas donation of oocytes or embryos can be technically quite simple, there are many psychological issues involved. As can be gathered from aforementioned discussions, the treatments developed for infertility appear to be somewhat illogical and in the style of "shot gun therapy." In the field of infertility, as in other areas of medicine, it is of paramount importance to know the details of disease mechanisms. This in turn will allow specific and logical treatments to be developed.

Key Words: In vitro fertilization; intracytoplasmic sperm injection; recent advances in assisted reproductive technologies.

Received March 20, 1998; Revised May 6, 1998; Accepted May 13, 1998.

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Introduction

Very few advances in medicine have received as much public attention, fascination, and concern as in vitro fertilization (IVF). This technology, together with similar procedures such as intrauterine insemination (IUI), intrafallopian transfer of gametes (GIFT), and oocyte and sperm donation, are all referred to as assisted reproductive technologies (ART).

Drs. Steptoe and Edwards developed the concept in humans of retrieving a mature oocyte from the ovary and fertilizing it in vitro to produce an embryo (in vitro fertilization [IVF]) and then transferring the embryo into the woman's uterus (embryo transfer [ET]). They reported the first pregnancy and childbirth from this procedure in 1978 *(1)*. While these authors retrieved the single oocyte that matured in humans naturally, the next advance in the field was to stimulate the ovaries with human menopausal gonadotropin (HCG) to be able to retrieve many oocytes. Several embryos could therefore be transferred, resulting in a better pregnancy rate *(2)*. With this advance came the problem of multiple births. This was partly overcome by the subsequent development of the technique of embryo reduction. At approx 7 wk *(3)* or 10–12 wk gestation *(4)*, some of the extra fetuses are either removed or destroyed. To retrieve the oocytes, the woman was anesthetized, and the oocytes obtained by aspiration through a laparoscope. The next major advance enabled the retrieval of oocytes under ultrasound guidance, without the need to do laparoscopy, or to administer anesthesia *(5)*. Cryopreservation allowed the extra embryos to be kept for future use *(6)*, although giving rise to the problem of clinics and hospitals becoming custodians of numerous embryos. Oocyte donation was developed on the same

line as sperm donation which allowed women without functional ovaries to conceive *(7)*.

In this review we discuss what has happened since the development of the aforementioned technologies, which have become well established and widely practiced.

Intracytoplasmic Sperm Injection

Technique of Intracytoplasmic Injection

Intracytoplasmic injection, without a doubt, is the most significant recent advance in ART developed to help couples with problems of oligospermia. Previously, nothing much could be done for oligospermic men, although numerous drugs and procedures had been tried. When such therapies were subjected to controlled trials, the benefits were very limited, if any.

In 1976, Uehara and Yanagimachi *(8)* discovered that a hamster oocyte can be surgically injected with isolated sperm nuclei resulting in egg activation with formation of both male and female pronuclei. Many other investigators have confirmed this and have demonstrated that surgical fertilization of mammalian ova can lead to functional capacity during fertilization *(9)*. Subsequently pioneer work at Jones Institute at the University of Eastern Virginia revealed that human spermatozoa that are incapable of penetrating and fertilizing ova in an in vitro system, are capable of decondensation and pronuclear formation if surgically injected into the cytoplasm of hamster oocytes *(10–12)*. This led to report by Lanzendorf et al. *(13)* in 1988 that microinjection of human spermatozoa into human oocytes can lead to pronuclear formation. In 1992, in a letter to *Lancet*, Palermo and colleagues *(14)* reported having treated patients who had failed to conceive with IVF with a new technique, which consisted of injecting sperm directly into the cytoplasm of oocytes at the metaphase-11 stage of development (intracytoplasmic injection [ICSI]). They reported the delivery of children conceived in this manner. The same authors reported a much larger series in 1993 *(15)*. The most encouraging aspect of this report was the very high pregnancy rate achieved with this method. The treatment group consisted of 150 couples who had either failed to have fertilization of oocytes in a previous IVF cycle, or the husband's sperm count was too low to be accepted into an IVF program. They reported 150 cycles of IVF with ICSI when, of the oocytes injected, 71% developed into embryos and only 10% did not have embryos for transfer. The clinical pregnancy rate (visualization of a gestational sac by ultrasound) was 35% per cycle, which indicated what was truly a breakthrough for this type of infertility. The women first received down regulation with intranasally administered gonadotrophin-releasing hormone agonist (GnRHa) buserelin, and again later, in association with human menopausal gonadotropin (HMG), to result in the production of several mature oocytes. Ovulation was induced with human chorionic gonadotrophin (HCG), followed by transvaginal oocyte retrieval. The luteal phase was supplemented with administration of intravaginal natural micronized progesterone. After oocyte retrieval, the oocytes were examined under an inverted microscope, and they were classified as mature, slightly immature, slightly hypermature, or immature. Subsequently, the cells of the cumulus and corona radiata were removed by incubation with hyaluronidase and the oocytes were searched for presence or absence of a germinal vesicle or the first polar body. Nuclear maturity was evaluated and the ooplasm was inspected. ICSI was carried out on all morphologically intact oocytes that had extruded the first polar body. Injection pipets were locally made by drawing thin walled glass capillary tubes using a microelectrode puller. The sperm was made immobile, just prior to aspiration into a micropipet, by exposing them to polyvinylpyrrolidone. Each oocyte was injected with one sperm with the sperm being released into the cytoplasm of the oocyte. The procedure was carried out under microscopic vision with the use of a dual micromanipulator with the oocyte being held with suction by one microtool and being injected by another manually operated micromanipulator.

Follow-Up of Pregnancies and Infants Born Through ICSI Procedure

Of course, the first concern about the development of such an effective technique was the health of the children born through such treatment. Bonduelle and colleagues *(16)*, in 1996, reported a "prospective follow-up study" of 423 children born after ICSI. The aim of the study was to evaluate karyotypes, congenital malformations, growth parameters, and developmental milestones. Prior to starting the treatment program, the couple would have committed themselves to a fairly rigorous follow-up of the fetus and their infant(s). Prenatal diagnosis was done either by amniocentesis or chorionic villus sampling in 238 of 320 pregnancies (78%). Abnormal fetal karyotype was found in only 1 of 238 studied (i.e., 0.3% abnormality rate). This karyotype abnormality was mosaicism 46 xx/47, xxx which occurred in a woman of 43.9 yr of age. There was an intrauterine death in this case. Four of a total of 293 (1.3%) cases had benign familial chromosomal structural aberrations, all of which were clearly shown to be inherited from the fathers and were therefore already present before conception and not attributable to the ICSI procedure. Neonatal complications of a major degree were encountered in 17 children of 420 live births. The major complications were sepsis, meningitis, major pulmonary problems, and intracranial bleed. Altogether, 9 infants died, of these 7 were from multiple births. Major malformations were found in 4 singleton children, 9 twin children, and 1 triplet child, which was at a rate of 3.3% (14/420).

From the outset, there has been an awareness that ICSI children should be evaluated for congenital abnormalities for several reasons. Through the process of ICSI we may

start an embryo from a genetically defective oocyte or sperm which would not have a chance to form an embryo naturally. A number of chemical and environmental elements are introduced directly into the oocyte, and these in turn may cause genetic defects. The natural timing of oocyte fertilization may be altered by ICSI. There is concern that this may lead to anomalies. However, a most encouraging finding was that the 3.3% malformation rate was similar to that of most of the national registries for natural pregnancies. This figure was also similar to the malformation rate of 3.6% reported for standard IVF *(17)*.

Since the initial success of ICSI for oligospermia and failure of oocyte fertilization, other uses for the procedure have been developed. One question was whether ICSI improved the success of IVF. In a prospective controlled randomized study, IVF with ICSI was compared with IVF alone for cases of infertility caused by fallopian tube disease. No difference was noted in the pregnancy rate *(18)*.

Obtaining Sperm from Epididymis for ICSI by a Surgical Technique

The next development was microsurgical epididymal sperm aspiration and ICSI for infertility resulting from congenital bilateral absence of the vas deferens. Twelve patients with this disorder were subjected to a procedure consisting of the administration of general anesthesia and unilateral hemiscrototomy. Using an operating microscope, the epididymis was dissected carefully and an epididymal tubule was opened by microscissors. The epididymal fluid was aspirated by means of a mouth-controlled, sterile, hand-pulled glass pipet. In this manner, motile spermatozoa were obtained for IVF-ICSI procedure. In all 14 patients, sperm was retrieved, and on 10 occasions, embryos were formed for transfer. This resulted in a 35.7% overall pregnancy rate per started trial and 50.0% pregnancy rate per embryo transfer *(19)*. This study showed that the above technique was highly effective, although, the sperm obtained were grossly abnormal with respect to motility and morphology. Sperm density ranged from 0.0001 to 81.5 × 10^6/mL (normal more than 20 × 10^6/mL) with 21.2 ± 6.7 × 10^6/mL sperm cells on average (±SE). Rapid progressive motility (type A motility according to the WHO) ranged from 0–4% and slow progressive motility ranged from 0–10%. In only 6 cases (43%) were progressively motile sperm cells recovered, and in only one case were rapid progressively motile sperm cells found.

Obtaining Sperm by Percutaneous Aspiration in Cases of Obstructive Azoospermia

The next advance was the simplification of the technique of obtaining sperm in cases of obstructive azoospermia by the use of percutaneous epididymal aspiration *(20)*. The previously described technique of microepididymal sperm aspiration with scrotal exploration under general anesthesia involved a certain amount of trauma and postoperative morbidity (i.e., pain, hematoma formation, and infection). Adhesions formation could make subsequent sperm aspiration difficult. Craft et al. *(20)*, reported their experience with the percutaneous technique in 20 patients with obstructive azoospermia. The causes of obstructive azoospermia were failed vasectomy reversal in 12 patients, congenital absence of the vas in 5, inflammatory obstruction in 2, and unknown in 1. In 16 patients (80%), they were successful in aspirating sperm. Of these, 3 achieved clinical pregnancy (18.8%), which is a fairly good rate for any IVF center. The procedure was very simple. A 21-gage butterfly needle, which was connected to a syringe, was used. The needle was directed into the head or corpus of the epididymis and sperm were aspirated. Craft et al. *(20)* recommended the open technique should the percutaneous technique fail.

The Use of ICSI for Male Immunological Abnormality

Another use of ICSI was reported for male immunological infertility by Nagy et al. *(21)*. It is known that antisperm antibodies can be present locally, in the semen, in the female genital tract, or in the systemic circulation, where they are directed against different types of sperm antigens *(22)*. Both can interfere with fertility and also reduce IVF results *(23,24)*. Antisperm antibodies directed against the head of the spermatozoon can interfere with sperm binding to the oocyte *(25)* and thus reduce the fertilization rate *(23)*; antisperm antibodies directed to the tail area of the sperm will impair the sperm motility resulting in reduced fertilization rate *(26)*. However, Collins et al. *(27)* failed to demonstrate any clear correlation between the presence of antisperm antibodies and reduced fertility potential. Nagy et al. *(21)* employed ICSI in 55 patients in whom the proportion of antisperm antibody-bound spermatozoa was 80% or higher as determined by the mixed antiglobulin reaction test. They found the mean normal fertilization rate to be 75.7% in these 55 cycles. In 26.4% clinical pregnancy was obtained and therefore Nagy et al. *(21)* proposed IVF-ICSI for infertility related to antisperm antibody.

Obtaining Sperm for ICSI by Performing Testicular Biopsy in Patients with Obstructive Azoospermia

In search of the spermatozoa the investigators went one step further by obtaining testicular biopsies in azoospermic men. Silber et al. *(28)* reported high fertilization and pregnancy rates after ICSI with spermatozoa obtained from testicular tissue. Their surgical technique for testicular biopsy was extremely simple. A 1-cm incision was made in the scrotal skin and carried through the peritoneal tunica vaginalis. After making a 0.5-cm incision in tunica albuginea, a small piece of the extruding testicular tissue was excised. The various layers were then stitched together. The testicular tissue obtained was finely minced in a special culture medium. Eventually, a few poorly motile or non-motile spermatozoa were identified and used for the ICSI procedure. Silber et al. compared the IVF–ICSI results

of spermatozoa obtained by epididymal aspiration with those obtained by testicular biopsy and found fertilization rates of 45 and 46%, respectively. The ongoing pregnancy rate in these series were 50 and 43%, respectively. Therefore, in the group of patients studied with congenital absence of the vas deferens or irreparable obstructive azoospermia, Silber and colleagues obtained excellent results with sperm from testicular biopsy if they were unsuccessful with epididymal aspiration.

Achieving Pregnancy from Acrosomeless Sperm by the Use of ICSI

Acrosomeless ("round headed") spermatozoa are unable to either penetrate the zona pellucida of the oocyte or fuse with the oolemma. Such sperm lack the acrosomal membrane and acrosine contents. This condition is believed to be hereditary, as there have been reports of brothers similarly affected. Lundin et al. *(29)* reported the case of a couple with the husband having acrosomeless sperm when IVF-ICSI was performed, resulting in a twin pregnancy.

Sperm May Be Obtained by Testicular Biopsy from Men with Azoospermia and High FSH Values Resulting in Pregnancy with the Use of ICSI

Infertile men with "sertoli cell only syndrome" show a complete lack of germ cells in their testicular biopsies. Such patients have small testes and normal androgenization. "Maturation arrest" is another cause of azoospermia, resulting from complete failure of reduction division, or meiosis, of tetraploid pachytene spermatocytes to haploid spermatids. Silber et al. *(30)* more recently demonstrated that if the entire testis is carefully sampled, more than half of the patients with "steroli cell only" or "maturation arrest" will have occasional foci of normal seminiferous tubules with spermatogenesis. Gil-Salmon et al. *(31)* reported achieving twin pregnancy in the case of a male with high follicle-stimulating hormone (FSH) values and biopsy diagnosis of "Steroli cell only" syndrome. Spermatozoa were obtained from a testicular biopsy. Gil-Salsom et al. showed that in male infertility with high FSH it is possible to achieve pregnancy with IVF-ICSI.

ICSI has been the most clinically applicable recent advance in ART. However, although it is a treatment for male disorders, the female has to bear most of the burden of the IVF treatment. Therefore ICSI should not prevent us from finding the cause of and more specific treatments for male infertility such that the therapeutic approach may be directed toward the male.

Simplification of IVF

There are problems with IVF that has limited its utilization and availability. There are dangers associated with the use of HMG including development of ascites, pleural effusion, thromboembolic disorders, respiratory and heart failure, and even death. The retrieval process can be lengthy requiring repeated injections of drugs that may lead to respiratory failure. The cost of the drugs and the procedure are high, making IVF inaccessible to many deserving couples.

Simplification of Ovarian Stimulation

In patients with tubal disease and in some of the younger patients, it may be possible to just use clomiphene citrate (CC) instead of HMG *(32)*. Thirty cycles of IVF were performed on 25 patients with an average age of 34 yr. The causes of infertility were tubal disease (64%), infertility of undetermined cause (28%), and the male factor (3%). The patients took CC orally from d 9 of the menstrual cycle. They started follicular monitoring from d 10, which was done on the average for 4 d, and then they received HCG 5000 iu on average at d 13. The average number of oocytes retrieved was 2; a total of 55 oocytes were retrieved with 82% fertilization. The average number of embryos transferred were 1.8, and a total of 7 clinical pregnancies and 6 ongoing pregnancies and deliveries were observed. There were no complications and there were only 2 twin pregnancies. All patients received intramuscular progesterone and estrogen for luteal phase support. With the use of cc alone, 16.3% fetuses were formed per oocyte retrieved. Clinical pregnancy and ongoing pregnancy or delivery per embryo transfer were 29 and 25%, respectively. Therefore a high pregnancy rate was achieved while avoiding many of the problems associated with IVF such as high cost, ovarian hyperstimulation, multiple births, or the need for cryopreservation of embryos.

Simplification of Oocyte Retrieval

The technique of oocyte retrieval has been simplified *(32)* so as to reduce the hazards and discomfort to the patient. The most elaborate retrieval procedure consists of using a full operating room facility and administrating general anesthesia with one or two embryologists present. The follicles are repeatedly washed until an oocyte is discovered. Khamsi et al. *(32)* referred to this protocol, which they had traditionally used, as the "long protocol." They compared it to their "short protocol" in which the follicles were sequentially aspirated into 50-cc syringes (containing heparinized, warmed, CO_2 equilibrated human tubal fluid type culture medium). The follicles were not washed and the oocytes were identified after termination of the retrieval by the same physician. The length of the procedure was 30–45 min for the "long" and 1–2 min for the "short" protocols. Much less medication was needed for the "short" compared to the "long" protocol (i.e., Fentanyl 50–70 mg and Diazepam 5–7.5 mg versus Fentanyl 50–250 mg and Diazepam 10–25 mg, respectively. There was no difference between the two techniques in either the number of oocytes retrieved or the clinical pregnancy rate. Therefore, Khamsi et al. *(32)* described a technique for oocyte retrieval that took only 1–2 min with reduction in hazards to the patient and reduction in cost.

Simplification of Tissue-Culture Technique

Khamsi et al. *(32)* modified the tissue-culture technique. In the "standard technique," the oocyte was aspirated into a test tube that was passed to an embryologist quickly to identify the oocyte. If no oocyte was found, the follicle was repeatedly washed. In the modified technique, the oocytes were directly aspirated into prewarmed syringes containing heparinized tissue-culture medium. Whereas in the standard method, each oocyte was cultured separately in a tube, in the modified method all oocytes were cultured in microdrops under oil. Oil provided a protective barrier and reduced the extent of CO_2 evaporation. In the standard technique the oocytes were stripped off their cumulus cells on the second day. In the modified technique no manipulation was done on the second day. The embryos were identified and transferred 2 d later, a procedure taking no more than 10 min, which can be done by a physician with limited training. By the third day, the cumulus has usually fallen off the embryo, and it is very easy to identify the embryos. The technique does not require stripping, which is otherwise done on the second day. Khamsi et al. found no decline in pregnancy rate with simplification of the technique.

How to Administer HMG More Carefully

The most clinically serious situation is encountered when the woman develops severe hyperstimulation syndrome. The major problem is that there is a large variation in response to injections of HMG from one patient to another. Attempt was made to predict the responsivity of patients on the basis of a study of 100 cases *(33)*. An index of responsivity was devised by dividing the level of estradiol at the day of HCG injection by the number of HMG ampules (75 IU each) used *(33)*. Khamsi et al. *(33)* found two factors that have profound effect on the responsivity index. One was the patient's weight. The 100 patients were divided into 3 equal groups, those weighing <55 kg, 55–67 kg, and >67 kg. The responsivity indexes were 559, 443, and 369 mmol/L/ampule, respectively. The other recognized factor was age. The 100 patients fell into 3 equal groups those aging <32, 32–36, and >36 yr. The responsivity index was 517, 453, and 368, respectively. Therefore, we now know that the thin, young woman is the one most sensitive to HMG and should be started on a low dose, whereas the older and overweight patient should be started on a higher dose. With simplification of IVF, safety of the patient is the first and most important and the first consideration in ART treatments.

Luteal Phase Monitoring

The luteal phase of the menstrual cycle in IVF patients has received much less scrutiny than the follicular phase. A comparison was made of four regimes for luteal phase hormonal support after ET in IVF cycles *(32)*. To evaluate efficacy of implantation, an index was devised: the number of gestational sacs seen (by ultrasound) divided by the number of embryos transferred. A greater number of gestational sacs per embryos transferred may be indicative of a better luteal phase support regime. The four types of luteal support were progesterone suppositories, progesterone injections, HCG injection plus progesterone injection, and progesterone and estrogen (estradiol valerate), both by injection. The implantation index (gestational sacs divided by the number of embryos transferred) were 0.04, 0.05, 0.06, and 0.09, respectively, indicating that combined estrogen and progesterone injection gave the best luteal support. If the pregnancy test was positive, the injections were continued until 8 wk after the embryo transfer, by which time the placenta was producing substantial amounts of estrogen and progesterone.

Cost Reduction

The high cost of performing IVF must be systematically studied and reduced to make IVF accessible to more people. Paying attention to these factors resulted in reducing the cost of all disposable materials for an IVF cycle to $84.00, without jeopardizing obtaining a reasonable pregnancy rate, i.e. 20% take home baby rate per ET *(32)*.

Assisted Hatching

Once a sperm enters the oocyte, the surrounding membrane, the zona pellucida, hardens that prevents the entry of more sperm (polyspermy), and may also be beneficial by protecting the embryo from immunological attack and aiding in oviduct transport. However, the membrane has to open up to allow the embryo to hatch for the process of implantation at the time of blastocyst expansion. Manipulation of embryos such as in vitro culture and cryopreservation may make the zona harder which may lead to prevention of hatching. In response to this problem, it was proposed that assisting the embryo to hatch by thinning the zona may lead to a better rate of implantation and greater success of the IVF cycle. Assisted embryo hatching (AEH) is not universally practiced in IVF centers, there are a number of reports that suggest it may improve pregnancy rate *(34–37)*. The authors in these studies have advocated the use of AEH in a selected group of patients:

1. Those with elevated maternal age (38 or more);
2. Elevated basal FSH;
3. Increased zona thickness;
4. Prior failure of IVF cycle(s);
5. Reduced embryonic cleavage; or
6. Excessive fragmentation of the embryos.

This procedure is performed with the same micromanipulation apparatus as was described for ICSI. The embryo at d 3 of culture is held by a holding micropipet. The zona is thinned with the use of an acidic solution (acidic tyrodes) that is temporarily brought into contact with the zona by an injecting micropipet. A defect equivalent to 1/3 to 1/2 of the diameter of an 8 cell blastomere is made on one side of the

embryo with the use of the acid. The embryo is then quickly removed from the acidic area. A randomized clinical trial performed in 1992 showed that this technique resulted in increased implantation *(35)*. The embryo transfer is performed in the afternoon of d 3 which is done under ultrasound guidance in some centers. Dramatic results were reported by Schlenker et al. *(38)* at the 1997 Meeting of the American Society of Reproductive Medicine. Patients 40 and over in age undergoing zona drilling (AEH) of all their embryos demonstrated a delivered pregnancy rate of 47% with an implantation rate (number of pregnancies divided by the number of embryos transferred) of 22%. The control group (no assisted hatching) revealed a delivered pregnancy rate of 11% and an implantation rate of 6%. These differences between the two groups were highly significant. A potential adverse effect is damage to the embryo by the acid tyrodes solution in the event that excess acid should enter the embryo through the defect created. Embryos may be more prone to damage during embryo transfer and therefore this must be done with care. The concern about creation of identical twins has not been substantiated. The results from various centers are different and this may be entirely related to the size of the hole being too small or too large and acid is not aspirated out of the perivitelline space or the embryo transfer has been traumatic.

Cytoplasmic Transfer into Human Oocytes

Some oocytes lack the ability to fertilize or to develop after fertilization, and women with such oocytes who wish to have a child require egg donation. It is possible that the problem with some of these oocytes is related to a deficiency in the cytoplasmic portion of the cell; that it is lacking the necessary apparatus or chemicals for development. Therefore, if cytoplasmic material from a normal oocyte were to be infused into the incompetent oocytes, there may be a chance to change the abnormal oocyte into a normal one *(39)*. Cohen et al. *(39)* treated three consenting couples in whom implantation had failed after numerous IVF cycles. Egg retrieval was synchronized between oocyte donor and recipients. Cytoplasm was removed from donated oocytes (normal) and injected into the perivitelline space of the recipient oocyte and then electrofusion was performed. Subsequently, sperm injection ICSI was performed. Of the 22 recipient oocytes treated, 21 (96%) fused with donor cytoplasm and were injected with sperm. Fourteen embryos were formed. Of these, 2 achieved pregnancy from cryopreserved embryos, but 1 miscarried. Cohen et al. established that cytoplasmically hybrid M11 oocytes can be fertilized normally after electrofusion, cleave and develop to the blastocyst stage. In a second group of 4 patients the authors had transferred the cytoplasm by microinjection *(39)*. Fourteen embryos were transferred in total and 3 patients became pregnant. One patient had a singleton and delivered, 1 has an ongoing pregnancy, and 1 miscarried. Cohen et al. reported the details of the first child born through this technique. Cytoplasm of the donor oocyte was aspirated into a pipet together with a sperm from the recipient's husband. Care was taken to aspirate the ooplasm from the egg pole opposite the polar body in order to avoid the oocyte metaphase spindle. Aspirated ooplasm and sperm were deposited in the patient's egg close to its metaphase spindle. In the 1 infant conceived through this procedure, amniocentesis was performed and confirmed the nuclear characteristics matching those of the parents. This excluded the possibility of transfer of nuclear material from the donor, during the cytoplasmic transfer. In conclusion, the report by Cohen et al. indicates that it may be feasible for some women with poor oocytes to have children of their own genetic material even though they must borrow cytoplasmic material from donor.

Working Out the Molecular Mechanism of Implantation

Much of the work of ART has been on the basis of "trial and error," or "random hitting," or "shot gun" therapy. Very little work has been done, or very few research grants have been available, for fundamental research into the basic molecular and cellular mechanisms so that such defects may be corrected by developing therapeutic modalities. As a result, many treatments seem illogical. For example, if a man is discovered to be oligospermic, the physician informs the couple that the cause of their infertility has been found, and it is a problem with the man. However, the burden and hazards of the solution, IVF with ICSI, will have to be borne by the woman. The public views this as illogical and "sexist" because we have devised a treatment for the man that can make the wife (be it rarely) very sick. The field of infertility lags behind many other disciplines of medicine in the development of an understanding of disease mechanisms. A fundamental study of biochemical mechanisms could lead to focused therapy, which would be more selective and much less hazardous and costly. The infertility specialist community must recognize this and convey it to the public and governmental authorities so that appropriate funds may be allotted for research and development in the area. One of the few areas in which some progress has been made in understanding the molecular mechanism of implantation in relationship to plasminogen activators (PAs). The work that was started on experimental animals and tissue-culture systems has been extended to humans *(40)*, which is a systematic and logical way of working out disease mechanisms so that targeted and focused modes of therapy may be instituted. Why some embryos do not implant and why once implanted some do not grow? In infertile women, the rates of implantation in IVF cycles have been reported to be anywhere from a few percent to about twenty percent. Why are so may embryos wasted? If there should be low estrogen or progesterone level the

embryos do not implant. If there is an immunological mechanism such as in systemic lupus erythematosus, again the embryos do not implant. Implantation is a complicated process requiring cell adhesion, cell invasion, breakdown of the intercellular barriers (to allow fetal tissue to be established), development of new blood vessels, and an immunological protection against the maternal defense mechanisms. Surely, all these require genes, and surely, the embryo may be missing one or several of these genes. In such cases, implantation will fail and as we stand now (without knowledge) we will not know why.

Plasminogen activators have been studied fundamentally, and then their roles have been established in humans *(40)*. This is an example of how the various genes can be discovered, to lead to developing therapeutic strategies in future. Some serine proteinases and metalloproteinases have been implicated in embryo implantation *(41,42)* because of their possible role in the trophoblast invasion process. These enzymes act by breaking down the basement membrane to allow implantation. PA, particularly the urokinase-type PA (uPA), is probably involved in such proteinase activities during embryo implantation. PA converts the inactive plasminogen into serine proteinase plasmin. This broad spectrum proteinase can cause degradation of the extracellular matrix directly or indirectly by activating latent metalloproteinases *(43)*.

A study was carried out on human preimplantation embryos *(40)* where PA activity was studied by a chromogenic assay and the uPA gene expression was studied by reverse transcription-polymerase chain reaction. Surplus oocytes and embryos were donated for this study by patients going through IVF cycles. The patients went through a standard IVF protocol and 2–4 cell stage embryos were included in this study. To determine PA activity in embryo secretion, two or three 2–4 cell stage embryos were cultured for 16–18 h. The embryos were further cultured for 3 d to obtain blastocysts. Subsequently the blastocyst embryos were cultured for another 16–18 h, during which the effect of adding recombinant human epidermal growth factor (EGF) was studied on their PA secretion. PA activity was not detectable in secretions by 2–4 cell embryos, whereas PA activity was detected in secretion by blastocysts. PA activity was studied during the last 16–18 h of the blastocyst culture, and showed a positive response to the addition of EGF in comparison to the controls. Khamsi et al. *(40)* verified that the blastocyst secretion was uPA. The gene expression for uPA was seen faintly at the 8–16 cell embryo stage but became readily visible at the blastocyst stage.

uPA is produced in experimental animals at the time of implantation and has been implicated in trophoblastic invasion (PA) *(44–46)*. In the study of Khamsi et al. *(40)* PA activity was found in secretions of blastocysts, and this was identified by zymography to be of the same molecular weight as uPA. It was demonstrated that uPA may play a role during implantation. Thus, in addition to uPA, some metalloproteinases are produced by human and mouse trophoblast cells, and are probably also important for the trophoblast invasion process *(42,47)*. A recent study showed that inactivation of tPA and uPA genes in mice by targeted mutagenesis reduced their litter size but did not completely prevent embryo implantation *(48)*. This finding may indicate that in addition to uPA there are probably other enzymes with similar functions. Many growth factors are implicated in implantation and some may act by producing proteinases such as uPA. The study by Khamsi et al. *(40)* showed that EGF increased uPA production in human blastocysts, concluding production of uPA in human blastocysts and its regulation by the growth factor EGF. This now can lead to the evaluation of uPA and EGF in human pathological conditions in which embryos do not implant. If there are conditions when the gene for uPA is lacking or the gene for EGF is missing, then the injection of such genes into these embryos may lead to implantation. Khamsi et al. are working toward development of specific and logical means of addressing the widely encountered problem of lack of implantation.

In Vitro Maturation of Immature Human Oocytes and the Achievement of Pregnancy

In vitro maturation (IVM) of immature oocytes and the achievement of pregnancy can potentially be a major advance in ART because it obviates the need for hyperstimulation of the woman, which can be hazardous and costly. The problem with ovarian hyperstimulation syndrome has been discussed. There is also concern that the repeated use of fertility drugs may increase the woman's chance of developing ovarian cancer *(49)*. Unfortunately, to date, application of IVM in humans has yielded very low pregnancy rates whereas it has become an established technique for farm animals *(50)*. The first pregnancy in humans resulting from IVM oocytes was reported by Cha et al. *(51)*. These authors aspirated oocytes from surgically removed ovaries (in patients who had to have one or both ovaries removed because of their own medical disorders) and obtained oocytes in various stages of maturity. They cultured the oocytes for 32–48 h. Two culture systems were used, one containing fetal cord serum and one containing 50% mature follicular fluid. They reported 36 and 50% maturity rate and 32 and 81% fertilization rates in the two culture systems, respectively.

The next group to report IVM of human oocytes leading to pregnancy was Trounson et al. *(52)*. Oocytes were retrieved from consenting patients in two groups, ovulatory women and those that were anovulatory as a result of polycystic ovarian syndrome (PCOS). They found the standard type of needle (for IVF oocyte retrieval) to be ineffective/unsuccessful in obtaining oocytes. Therefore, they designed a new needle that was shorter in length, had shortened bevel, and was more rigid. For most patients, they used ultrasoni-

cally guided oocyte retrieval. In those for which this could not be achieved, they resorted to laparoscopic oocyte retrieval. They reported the pregnancy and birth of one child after 42 cycles of treatment in 42 patients, i.e., a pregnancy rate of 2% per patient. The nature of the culture medium is probably the key factor contributing to the success of an IVM program. However, the authors found no benefit from adding 50% mature human follicular fluid or 50% human peritoneal fluid. They based the composition of their culture medium or media they had successfully used for this purpose in cattle *(53)*. The timing of insemination of the oocytes was considered to be important. They inseminated the oocytes at 29.5–32.5 h and 34.5–35.5 h after retrieval, and found better results with the latter. In other studies, the authors did not find better results when insemination was performed at 48 h. However, Cha et al. *(51)* inseminated at 32–48 h of culture. The presence of hormones *(54)* and growth factors may be an important ingredient in such culture media. Zhang et al. *(55)* studied the fate of immature oocytes retrieved from standard IVF cycles. In a prospective randomized study, they showed that the addition of HMG (Pergonal) improved early human embryonic development. They showed that special treatment of immature oocytes in this manner may lead to a higher number of embryos becoming available for transfer. An increased fertilization rate was reported where granulosa cells were added to the culture medium used to mature oocytes *(56)*, although Trounson et al. *(52)* did not find this to be the case in their studies. Gomez et al. *(57)* showed that the addition of EGF and insulin-like growth factor (IGF1) can enhance the development of immature oocytes maturing in vivo. Indeed, there is a great deal of experimental interest in the study of IGF1 and its analogs in oocyte maturation *(58,59)*, and it will have to be further studied in humans. Trounson et al. *(52)* indicated that in vitro maturation of oocytes in patients with PCOs may eventually be superior to other methods of treatment such as ovarian electrocoagulation *(60)* or ovarian wedge resection *(61,62)*. These latter two techniques may lead to de novo adhesion formation in 17–27% or more of the patients.

Nagy et al. *(63)* pointed out that immature oocytes cultured in vitro may experience hardening of zona pellucida, resulting in lack of fertilization. They reported the clinical findings of one IVF patient from whom many immature oocytes were retrieved. These oocytes were cultured for 30 h (compared with approx 6 h in standard IVF), and then were inseminated by direct sperm injection. From 14 oocytes treated in this manner, 4 embryos were transferred and 1 singleton pregnancy was achieved. Determining whether or not ICSI should be practiced routinely for fertilization of immature oocytes (matured in vitro) will require a controlled study. There has been a great deal of interest in in vitro oocyte maturation and many new innovative approaches have been published. Volarik et al. *(64)* showed that the age of the donor is important. Herbert et al. *(65)* showed the development of calcium signalling mechanisms during maturation of human oocytes. Park et al. *(66)* made observations on the spindle configurations of human oocytes matured in vitro. Farhi et al. *(67)* noted that incubation with sperm enhances in vitro maturation of oocytes from the germinal vesicle to M2 stage. However, it is to be realized that in vitro maturation of immature oocytes has not reached a level of clinical application as of yet but similar technology is very successfully used in farm animals.

Psychological Aspects of Gamete Donation on an Altruistic Basis

Organ donation in some countries, such as Canada, has been practiced universally on an altruistic basis; no financial or other rewards have been expected or offered. For example, the complete blood transfusion service in Canada is run on this basis, whereas in the United States, it is partly on altruistic basis and partly on a paid basis. When it comes to donating a kidney, the society finds it unacceptable that a person should sell his or her kidney, whereas voluntary donation of a kidney with no reward is practiced and revered. In both Canada and the United States the sperm donors have usually been paid a small sum of money, although a study of such donors has shown the presence of an altruistic element. Both countries did not appear to be too worried about such payments. A similar gamete donation program has become available for oocytes, but a major problem has been the availability of oocyte donors. Originally, some authorities had discouraged the use of know donors for fear of future emotional and legal complications. Furthermore, oocyte donation requires that the donor be treated with medication and subjected to the process of oocyte retrieval, all of which are associated with morbidity and even very rarely mortality. Infertile couples in search of oocyte donors began advertising for oocyte donors and offered money far in excess of what sperm donors were receiving. This inspired outrage in some members of the public being concerned that this was yet another way of exploitation of a woman's body. Therefore, a law has been proposed in Canada banning all forms of gamete donation except on an altruistic basis. However, gamete transfer between people known to each other may potentially produce new complications that do not exist if the donor and recipient never come into contact. Some of these psychological issues are now being systematically studied by Endman and colleagues *(68)*, and their experience is being made available. The authors noted that each of the parties involved in oocyte donation, the recipient, her partner, the donor, and the potential child, have their own feelings, motives, and needs with respect to the program. All these aspects must be considered as they result in a diverse set of interpersonal relationships and psychological issues facing those individuals. The authors studied three major areas: motivation, disclosure and anonymity, and support systems

and emotional response. With respect to motivation, none of the 10 donors studied expected any financial reward. All 10 felt a sense of fulfillment in being able to help a family member. Seven of 10 indicated that participating as an oocyte donor was analogous to offering a gift ("a gift of life") and all 10 donors felt they were helping someone by being an oocyte donor. On the subject of anonymity and disclosure, 8 of 10 donors had not informed any other family member or friend about their plan to be an oocyte donor. Regarding disclosure to the child, 8 of 10 recipients felt they may not tell the child; the other 2 were uncertain. Pertaining to emotional support 7 of 10 donors thought they had excellent support; the other 3 felt they had good support. The corresponding figures for the recipients were 6 and 4, respectively. When the donors were asked whether they anticipated any feelings of responsibility toward the child, 9 indicated "no" and 1 was uncertain *(68)*.

The question of motivation has been discussed by several other authors in the context of other national laws and cultures. They have also found that altruism and a sense of helping others has been a major motivating factor, even in programs in which a financial reward is offered *(69–72)*. Another controversial issue is donor anonymity. The question facing the donor is how fully friends and family should be informed of her intention. In a study by Baetens et al. *(73)*, the majority of couples involved in a donor oocyte program had decided not to tell anyone and also not to inform the child conceived through treatment. However, in patients reported by Kirkland et al. *(70)* and Pettee and Weckstein *(74)* the donor and recipient told one or several or all other members of the family and friends. It is of interest to note that Klock and Maier *(75)*, reported that in a therapeutic donor insemination program, 81% of the donors who had confided in one or more people regretted having done so. Regardless, individuals involved in an oocyte donation program benefit from support before, during and after treatment. Studies by Pettee and Weckstein *(74)* and Sewell and Mason *(76)* emphasized the need for and importance of developing a support network and having available counselling in oocyte donation programs. In the study of Khamsi and colleagues *(68)* all parties had full satisfaction with the concept that the oocyte donor was known to the recipient couple. However, the authors provided counselling by a person with substantial experience in the field. In the context of their series for the length of the study, the triad of oocyte donor, oocyte recipient, and her husband appeared well satisfied. This uniform satisfaction may well be related to patient selections. More work is needed to ascertain:

1. What is the percentage of such couples who may benefit from oocyte donation by a known donor?
2. What are the long-term consequences of such knowledge among the triad?
3. What are the signs we have to look for in advance to avoid the situations in which such a program may fail?

Conclusion

We have reviewed recent advances in ART with the humble acceptance of the fact that many worthy publications and subjects are left out. The whole area of ART for the prevention of genetically transmitted diseases, for instance, has not been discussed, because this would have required an exhaustive review of genetic disorders. The technology of IVF and embryo biopsy are quite well established. An attempt has been made to give the reader a vision of where the field has to go to be more acceptable by the society. Embryo cloning, childbearing by menopausal women, and turning human couples into producers of "rodent type" litters are not considered by us to be advances in the field, and therefore have not been covered. As "health providers" in the field of infertility, we have our usual patients in mind. These are couples who have delayed having a family so as to ensure that they would be well equipped to take care of their children. Totally unexpected to them, this delay has reduced their fertility.

This review has also paid particular attention to effort made to reduce the hazards to women caused by such treatments. Short-term problems of ovarian hyperstimulation have been repeatedly addressed, because this is a hazard with which all in the field have been faced. Similarly, the long-term effect of the repeated use of fertility drugs on the woman's body, including risk of increased ovarian cancer *(49)*, has been a concern; we have therefore reviewed, in detail, methods of maturing oocytes in vitro (without the use of fertility drugs for the woman). Higher success rates per trial obviously mean less hazard and lower cost, and therefore we have reviewed original works which address this question, and have reported success rates in excess of 25% per trial. These included ICSI, AEH, and ET at the blastocyte stage. The cost of the procedures have been prohibitively expensive. Therefore, we have systematically reviewed the advanced that have lead to cost reduction *(32)*. We have been sensitive to public opinion and acceptability of ART to various communities. Most couples prefer to use their own gametes over donated ones, and some find gamete donation to be totally unacceptable for religious or ideological reasons. Therefore, we have emphasized IVF-ICSI that obviates the need for sperm donation for a large number of patients who, until recently, would have been offered sperm donation.

The couples who go through ART have the inherent psychological burden of infertility plus the new stresses created by treatments. For example, drugs such as CC may have profound (though transitory) psychological effects in some patients. Therefore, we have given the investigative work done in psychological evaluation a prominent place in this review. We have been sensitive to the widely held public perception that gametes, as human organs, should not be traded, but be offered altruistically. Therefore we have given prominence to health care providers, especially

the psychologists, who are addressing the problems that may arise when the donor and recipient know each other and will live in proximity with the knowledge of the genetic inheritance of their children *(68)*.

This review may also allow the reader to have a new perspective as to the cause of infertility. It is now possible to "work backwards" from knowledge of effective treatments to the reasons for infertility. The male factor is reported to be the sole reason for infertility in one third of the infertile couples and a contributing factor in another third. A large percentage of such problems are now overcome by ICSI of the sperm into oocytes. Therefore, this means that a major factor in male infertility is related to the lack of ability of the sperm to move, penetrate the zona pellucida, and fully enter the cytoplasm of the oocytes. This latter step is important because subzonal placement of sperms is much less beneficial than actually depositing the sperm inside of the cytoplasm of an oocyte. As the information becomes available for repeated IVF-ICSI use for such patients, we will know what percentage of male factor infertility can be cured with IVF-ICSI. Oocytes from various patients have different potencies with respect to fertilizability, formation of embryos, and viable fetuses. One determinant is the chronological age of the oocytes and another is the functional age of the ovaries. Gradual failure or early failure of oocyte function can be determined by the rise of d 3 FSH estimation in the woman's blood. With gradual failure of ovaries the FSH gradually rises. What is the mechanism for this failure of oocytes? It was shown that performing zona pellucida drilling of the embryo and AEH may improve the chance that an embryo will implant *(36)*. Therefore, the hardening of the zona pellucida of an aging oocyte may be the only reason why a fertilized oocyte may not implant as the embryo cannot hatch.

Embryo implantation requires many active steps such as cell invasion and new vessel formation. These are under genetic control and are influenced by various growth factors. For example, uPA is one of the proteinases necessary for cell invasion, which in turn is influenced by the EGF *(40)*. It is possible that the embryo may be defective in such genes, and this may be the reason for infertility. It may eventually be possible to inject such a gene into the embryo, before the embryo is transferred, providing a targeted and specific treatment for the infertility of the couple. Do such embryos originate from parents that both lack the gene, and in such situation will sperm donation be the answer? "Working backwards" from effective treatment to the cause of infertility, we are now arriving at some of the answers which have eluded us in the past.

Prominence has been given to the systematic study of embryos and infants in an IVF-ICSI program *(10)*. Such detailed studies are absolutely essential for us to know, as soon as possible, if any problems are developing. It should not be forgotten that the use of diethylstilbestrol did not show its serious consequences until one generation later, when the female offsprings of such mothers showed serious health problems. The number of children born through procedures such as IVF-ICSI are going to be limited and it should be possible for the health of such children to be monitored indefinitely and reported to a central registry.

Acknowledgments

The authors acknowledge Angela Sammut for her help in the preparation of this manuscript. The authors also thank Kathryn Khamsi for her help in editing this manuscript.

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