Experientia 41 (1985), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Biological aspects of in vitro fertilization

by R. J. van Kooij, M. Balerna and A. Campana

Gynecological Endocrinology Unit, 'La Carità' Hospital, CH-6600 Locarno (Switzerland)

Key words. In vitro fertilization; spermatozoa capacitation; oocyte maturation; sperm-egg fusion.

Fertilization and its preliminaries

In vitro fertilization and embryo transfer has not only been the major breakthrough in the alleviation of infertility in the last decade, it has also made possible the gathering of essential biological information on the process of fertilization in the human. The presence in the laboratory of human eggs, interacting with spermatozoa under the eyes of the investigators, has given rise to a burst of articles in scientific periodicals.

It is the purpose of this review to give a short compilation of the most important achievements of the last years on this subject.

A) Acquisition of fertilizing ability by spermatozoa

Already in the 50s it became clear that epidydimal or ejaculated spermatozoa need to undergo additional maturation before fertilization is possible [9]. This process was called capacitation[3]. Capacitation probably involves a series of changes at the molecular level, and these remain today unidentified and cannot therefore be assessed directly. We have to rely on its outcome, i.e. the capacity of the gametes to fertilize, to identify its occurrence.

After the spermatozoa have been capacitated they can undergo the so-called acrosome reaction[5]. This involves a vesiculation process between the outer acrosomal membrane and the overlying plasma membrane (fig. 1), and results in the liberation of acrosomal enzymes (e.g. acrosin, hyaluronidase, neuraminidase, arylsulfatase). In addition, the inner acrosomal membrane becomes the new outer membrane around the head of the spermatozoon. This vesiculation process seems to be essential for fusion to take place between the oocyte membrane and the spermatozoal membrane[6]. At which point during the sequence of events leading to fertilization the spermatozoon loses its acrosome is as yet unknown.

To penetrate the oocyte, spermatozoa have to negotiate several cellular and acellular layers around the oocyte (cumulus matrix, corona radiata and zona pellucida). An active role of acrosomal enzymes during the penetration process has been suggested[22,53], but the precise role of these enzymes remains obscure. At least the penetration through the cumulus matrix seems, however, to be independent of acrosomal enzymes, since round-headed, acrosomeless spermatozoa have been found to have penetrated deeply into the cumulus[37]. Some investigators believe that fertilizing spermatozoa initiate the acrosome reaction during cumulus penetration but before reaching the surface of the zona pellucida[13]. Others[20] concluded that the acrosome reaction of the fertilizing spermatozoon begins at the surface of the zona pellucida. Some aspects of the process of attachment to the zona during the in vivo fertilization process in the hamster have been described recently by Yanagimachi and Philips[68]. In their experiments done with oocytes removed from the ampullar part of the oviduct after natural mating, these investigators observed that remnants of the acrosomal cap were attached firmly on the zona surface. These remnants were split open by the sperm heads and left behind as the sperm advanced in the zona. How the different membrane systems (plasma membrane, vesiculating acrosome, inner acrosomal membrane or equatorial segments) are involved in the pre-penetrative binding process is not yet known.

As for penetration of the human zona pellucida, it should be mentioned that Lopata[37] observed a spermatozoon which had penetrated deeply into the zona, with an acrosomal cap which was at least partly intact. Also Stambaugh[52] described a monkey spermatozoon with acrosomal components within the zona pellucida. Since zona penetration without vigorous motility seems impossible this parameter must be considered as well as the status of the acrosomal cap and possible enzyme activity. In some rodent species a hyperactivated motility pattern has been described during penetration of the egg investments[65]. For human spermatozoa no such hyperactivation has yet been reported. However, special modes of sperm movement, possibly related to fertilization potential, have been described by Aitken[1].

Concerning capacitation, acrosome reaction and zona penetration it must be kept in mind that species-specific mechanisms are perhaps operating, complicating our picture of the preliminaries of fertilization.

Another important point to keep in mind is that in vivo and in vitro fertilization may differ in many aspects.

During in vivo fertilization in the human it has been estimated that only a few hundred to a thousand active spermatozoa are present in the ampulla during the periovulatory period[40]. In the hamster, Cummins and Yanagi-

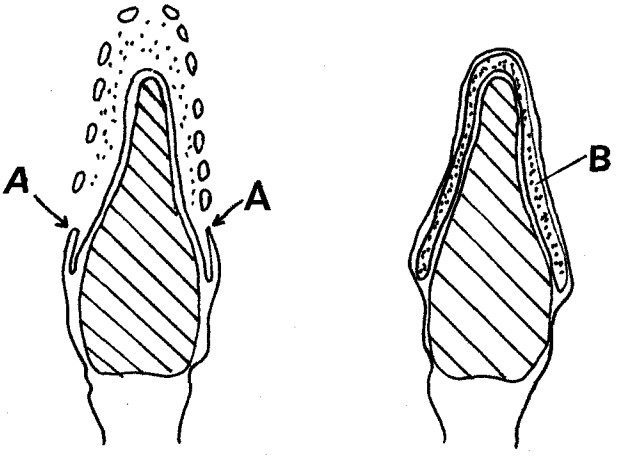

Figure 1. Drawing of the head of an intact spermatozoon (right) and a spermatozoon after the acrosome reaction has taken place (left). Arrows at *A* points of fusion between the acrosomal membrane and the original plasma membrane. *B* intact acrosome before reaction.

machi[13] have discovered that the number of spermatozoa within the ampullary region of an oviduct of a naturally ovulating female does not exceed the number of eggs in each ampulla until 50% or more of the eggs have been fertilized. In striking contrast is the number of spermatozoa used per oocyte in in vitro fertilization procedures. Although the numbers of around 10^6 spermatozoa/ml medium, used in the 'early days' of IVF, now seem too high, a lower limit of 50000 active spermatozoa per ml has recently been suggested[11,71]. Around 10^5–2×10^5 spermatozoa are used currently for in vitro fertilization. From such data it may be concluded that spermatozoa able to fertilize in vivo represent a selected, more fertile proportion of the more than 10^8 spermatozoa ejaculated normally. Indeed, a selection process operating at the level of the cervical mucus or the utero-tubal junction has been described[41]. Also, the possibility that the capacitation in vivo proceeds in a different, maybe more efficient way from that in vitro cannot be excluded. It is known that Ca^{++} plays an essential role in the steps leading to fertilization[69]. During capacitation, molecular alterations in the membrane systems of the sperms permit an increased influx of Ca^{++}, culminating in the expression of fertilizing ability[24].

Epididymal and endometrial enzymes[31], albumin[14] or hormonal stimuli released into the tubal fluid around the time of ovulation[39], have been implicated in such membrane changes. However, in view of the scanty knowledge of these phenomena more research needs to be done on the regulation of the Ca^{++} influx and on the molecular basis of the capacitation process. The use of in vitro systems should permit the identification of potential key steps in the process leading to fertilization.

B) Sperm-egg fusion

Once the sperm has reached the perivitelline space the next step is to induce fusion between a specialized membrane region and the vitelline membrane. The fusogenic region might be the postacrosomal membrane region[67] or the equatorial segment[7]. However, no general agreement on this item exists today. It is worthy to note in this context that already in 1975 Soupart and Strong[50] found that acrosome-intact human spermatozoa can penetrate zona-free human oocytes which were matured in vitro. On the other hand, fusion of human spermatozoa with zona-free hamster oocytes only seems to occur after an acrosome reaction has taken place[54]. The data of Soupart and Strong need confirmation by experiments with mature human oocytes before we can reach any conclusion concerning possible essential difference between the species on this point.

After membrane fusion between sperm and egg, complete incorporation of the spermatozoal contents in the egg ooplasm occurs. Probably, as found in the lower species[18] the fusion process initiates a complex cascade of events resulting in the following: 1) depolarization of the oocyte membrane, 2) onset of the cortical granule reaction (thought to be responsible for zona reaction and the blocking to polyspermy) and 3) reinitiation of the second meiotic reduction division[36].

The cortical granules, released after fertilization, have migrated to the periphery of the egg in the final maturation phase. These vesicles are thought to contain enzymes or proenzymes possibly of a trypsin-like nature[23], able to interact with the zonal glycoproteins. A physicochemical change in the zona, which prevents further sperm penetration, has been reported to result[62].

After fertilization, the second meiotic division with formation of the second polar body seems to be virtually completed within 3 h[36]. During this division maternal non-reduction can occur leading to triploidy, a condition usually leading to a spontaneous abortion. Before complete detachment of the second polar body the oocyte seems to undergo a phase of contraction which makes the cytoplasm occupy only about 50% of the intrazonal volume[37]. The ooplasm appears to be fully reexpanded by the time the pronuclei are well formed. This formation of pronuclei can be described as the next step in the fertilization process; the nucleus of the fertilizing spermatozoon begins to be dismantled by factor(s) in the ooplasm, and 6 h after fertilization the male and female chromatin have developed into small spherical structures, usually widely separated from each other. By 18 h the male pronucleus seems somewhat larger than its female partner and both are approaching each other to prepare for syngamy. The first mitotic division may occur 24–28 h after insemination[37]. The molecular mechanisms underlying the decondensation of sperm chromatin are still unknown. Decondensation can be observed also after fusion of human spermatozoa and zona-free hamster oocytes, a recently developed biological test-system for the screening of male fertility (see review in Yanagimachi[66]).

Methodological aspects of in vitro fertilization

A) Media

Several media have been successfully used for the culture of human gametes: Earl's medium[16], Ham's F 10 modified by Lopata[34], Whittingham's T6, Trounson[59], HTF[47]. Protein supplementation mostly takes place with serum of the patient or umbilical cord serum. The use of pure patient serum, detoxified at 56 °C and supplemented with antibiotics[29], has also been reported to be successful for all steps in IVF.

The rates of fertilization and embryo division do not seem to vary essentially between media, but pregnancy rates have been reported to be better when cord serum is used as a supplementation[33]. There is also a report claiming that HTF (a medium with a somewhat higher potassium/sodium ratio) gives more pregnancies per embryo transfer than Ham's F 10[47].

B) Oocyte maturity

To achieve normal fertilization and normal development, fully mature oocytes are needed. Insemination of an immature oocyte may lead to abnormal fertilization or impaired embryonic development, if any occurs. Neither the completion of the first meiotic division nor the activation of the characteristic ovulatory cumulus dispersion necessarily indicates a completion of oocyte maturation[59]. It is not yet possible to determine precisely the state of oocyte maturation with noninvasive techniques. Oocytes of different degrees of maturity are found after aspiration from

their follicles at laparoscopy. This is a result of the exogenous hormonal stimulation[21].

Secondly, there is a considerable asynchrony between oocyte maturation and follicular maturation[35]. This means that biochemical analysis of follicular fluid can only partly be of help in the assessment of egg maturity. However, significant differences have been described in the biochemical composition of follicular fluids from cases in which the oocyte cleaved and produced a pregnancy and from those where this did not happen. Isoelectric focusing patterns of the follicular fluid proteins revealed, e.g. differences in the a_1-antitrypsin content between atretic and healthy follicles[44]. Also, the quality of the cumulus clot surrounding the oocyte seems to have some predictive value. In general the cleaving potential of oocytes with a large, stretchable cumulus was found to be better than that of oocytes which had a small cumulus upon aspiration[56]. It has been recognized that completion of oocyte maturation after aspiration can be achieved by further culture in vitro before insemination takes place; if the oocytes are cultured for 5–6 h after clinical recovery, fertilization and normal development are significantly improved[60].

After such a culture period Sathananthan and Trounson[48] found an increase in the number of cortical granules at the egg periphery. This accumulation might reflect the additional maturation of the oocyte, necessary for normal development after fertilization. Also, oocytes which still have a germinal vesicle may be cultured in vitro to maturity.

In such cases culture periods of up to 30 h are needed. These oocytes can then be fertilized, albeit with a somewhat lower success-rate, and can result in normal pregnancies[61]. How long the oocyte remains fertilizable after it has reached full maturity is not known exactly. In many animal species a delayed fertilization results in polyspermy[4,27].

In our own work an oocyte recovered from the peritoneal fluid, after spontaneous ovulation had taken place, showed three pronuclei after insemination in vitro. Nevertheless, Fishel et al.[19] reported normal fertilization and development with oocytes inseminated after an unwanted delay of 23 h.

C) Sperm preparation

The removal of seminal plasma is thought to be essential, because traces of it seem to inhibit the onset of fertilizing capacity[28]. This can be done by repeated mild centrifugation and resuspension. Three such washing steps are considered to be effective[60]. An alternative approach is to layer medium carefully over the original semen or on the washed sperm pellet and to leave this system for different periods of time to allow the motile sperms to swim into the medium layer. If enough motile sperms are present, this method can be very effective in removing leukocytes, dead spermatozoa and debris. Several contributions describing such methods have been published in the last two years[11,55,71].

What follows is a short account of the procedure used in our laboratory. After liquefaction of the semen, an aliquot with at least 10×10^6 motile sperms (if possible we use $40–50 \times 10^6$ sperms) is taken, diluted 5 times with medium and centrifuged for 10 min at $300 \times g$. The pellet is resuspended with medium to the previous volume and again centrifuged. The pellet is then resuspended in 0.5 ml of pure serum (i.e. the same serum (detoxified at 56 °C) used for medium supplementation). This suspension is carefully overlayered with 1.0 ml of 10% serum supplemented-medium, placed at an angle of about 30° to enhance the surface contact area, and left in the CO_2 incubator at 37 °C for 1–2 h. Then an aliquot is removed from the upper (medium) layer to evaluate number and motility. Finally, an aliquot of medium containing 3×10^5 spermatozoa is added to the oocytes after removing the same amount of fluid from the culture dish containing the oocytes. The total volume in which oocytes are incubated is 3 ml, resulting in a final sperm concentration of 10^5 spermatozoa/ml.

Such a layering method enables us to obtain suspensions of highly mobile sperm cells which have undergone a process of self-selection. In comparison to the original sperm population, these suspensions are normally found to be enriched with respect to sperm motility and morphology (table 1). In addition, leukocytes and the major part of the bacteria and debris present remain in the lower layer. By this two-layer system the selection process which takes place in vivo is also mimicked.

In cases of severe asthenozoospermia, spermagglutination due to immunological factors, or combined oligo-asthenozoospermia one may lose too many sperm cells during the sample preparation. In such cases we have to rely on the simple centrifugation procedure outlined above, possibly followed by some incubation to achieve sedimentation (by simple gravitation) of cellular debris and nonmotile spermatozoa. Recent research[11] suggests that even in cases of severe oligo-, astheno- or teratozoospermia fertilization and pregnancy is possible. Also the presence of (at least certain types of) antispermatozoal antibodies does not seem to preclude fertilization and pregnancy[70].

D) Embryo culture

6–18 h after insemination the sperms are removed from the culture by transferring the oocyte to new medium. To visualize the pronuclei and polar bodies the corona cells still adhering to the zona may be dissected off with fine needles or pipetted away with fine glass pipettes. It is supposed that in vivo cumulus cells disappear in the mammalian oviduct by the action of the cilia[25]. After the corona cells have been removed, the pronuclei are readily visible with a reversed microscope. Abnormally fertilized eggs (triploidic or more) can undergo a regular and quick cell division. This means that polyploidy should be diagnosed before the first cell division is completed (normally beyond 24 h after insemination[8]).

Table 1. Sperm recovery for in vitro fertilization

	I	II	
% normal sperms	40.2 ± 4.3	52.4 ± 4.2	$p < 0.01$
% motile sperms	52.8 ± 8.3	83.8 ± 4.1	$p < 0.005$

Improvement of the quality of sperm suspensions after application of the two-layer selection system. Under I the values in the ejaculated semen are given, under II the values after layering are presented. Significance is calculated via Wilcoxon's rank test for paired samples.

After confirmation of fertilization one inspects the oocytes at another time within the next 24 h and once again at transfer, which takes place within 48 h after insemination. The cleavage intervals of the fertilized oocyte have been described by Edwards et al.[16] and by Trounson et al.[60]. Clearly this interval is shorter than 24 h because observation of pronuclei at 20 h after insemination can be followed by the observation of a four-celled embryo 24 h later. It has been reported that rapidly cleaving embryos seem to have better chances on implantation than the slower ones[37]. Some cleavage phases are shown in figure 2. There are currently no good standards by which to judge the quality of a developing embryo with respect to its potential to develop into a fetus. Fertilized oocytes may divide very regularly with equally sized blastomeres, but they may also have cytoplasmic blebs and uneven sized blastomeres, with one or more blastomeres becoming arrested during growth[19,64]. Both conditions can result in normal pregnancies.

Morphological standards are thus inadequate to decide which embryo to transfer and which not, in cases where too many are available for transfer. There is an obvious need for noninvasive techniques by which one could assess embryo quality before transfer. This in turn probably means that experiments on pre-implantation embryos will be necessary to correlate metabolic pathways, morphology, chromosome make-up and, eventually, excreted 'early pregnancy factors'.

E) Embryo transfer

Replacement of the embryos grown in vitro into the uterus occurs transcervically. It is a simple and quick procedure if performed by an experienced physician. The

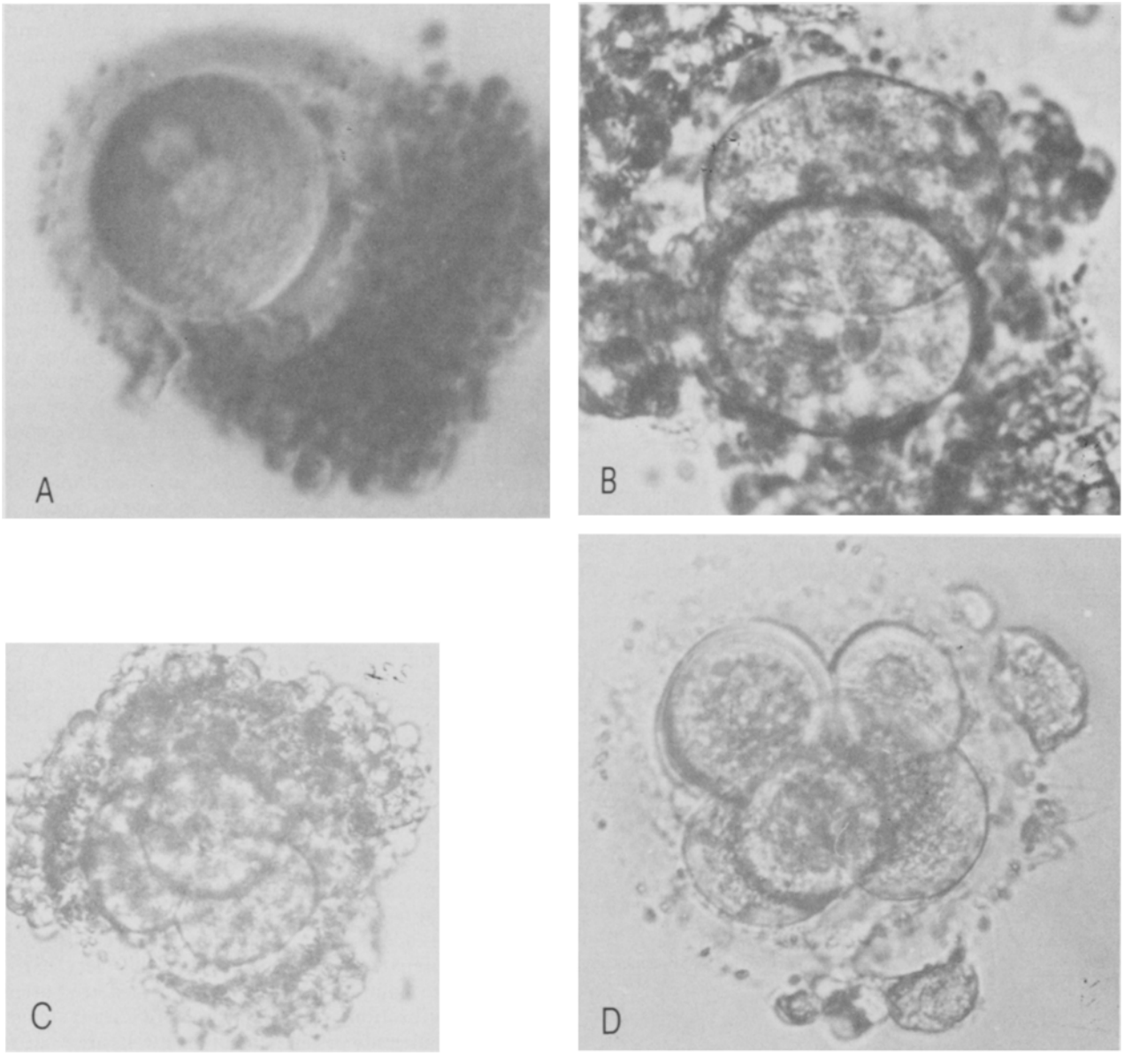

Figure 2. Photographs of some cleavage stages of human fertilized oocytes taken during the in vitro fertilization program of the authors. *A* fertilized oocyte (two pronuclei are visible); *B* two-celled stage (approx. 20 h after insemination); *C* four- celled stage (approx. 40 h after insemination); *D* six- to eight-celled embryo (approx. 48 h after insemination).

aim is to deliver the embryo(s) atraumatically near the uterine fundus. The medium in which the embryo is carried to its place varies from team to team. Sometimes culture medium is used, sometimes the culture medium is enriched up to 75% with patient's serum, sometimes HEPES is added to stabilize the pH of the medium during the handling period outside the incubator[46]. Comparing culture medium and a 1:1 mixture of serum and culture medium, Nayudu et al.[44] concluded that the latter resulted in more pregnancies per transfer. The procedure varies in a number of other details between the teams. A description of several aspects is provided by Jones et al.[26] and Trotnow et al.[58].

Pregnancy rates after embryo transfer

A) Simultaneous transfer of more than one embryo

In the various IVF/ET centers the pregnancy rate has risen substantially since the first birth in 1978[17]. The procedure has developed into a widely accepted infertility treatment, which already has success rates comparable to conventional treatments like tubal surgery. The use of the natural cycle for ovum pick-up, as formerly advocated by the group of Edwards and Steptoe, has largely been replaced by stimulated cycles, using Clomiphene, human menopausal gonadotropins, 'pure' FSH or combinations thereof. This ovarian stimulation results, in many patients, in the generation of more than one embryo available for transfer. The practice of such 'multiple embryo transfer' is probably the most important factor contributing to the better pregnancy rates in terms of live births per treatment cycle. In table 2 we have given a compilation of data from the literature. Since single transfers in the natural cycle or the stimulated cycle give almost equal pregnancy rates, the natural cycle has no advantage above the stimulated one in terms of pregnancy success, but only disavantages in terms of management of patients and around-the-clock availability of staff people. On the contrary, the pregnancy rate in the stimulated cycle rises when two or more embryos are transferred simultaneously, reaching the rate of 31% when four or more embryos are transferred, which is clinically very acceptable indeed (table 2). The total number of embryos transferred in these five studies was 3429 from which 445 implanted (12.9%).

B) Viability of the embryo after IVF

Theoretically, the chance of an embryo implanting after its transfer into the uterus is dependent on a large number of factors which may be characterized as uterine and embryonic factors. The receptivity of the uterus for a healthy embryo is dependent not only on endocrine environment and the response of the endometrium to it, but also depends on the quality of the embryo transfer procedure (is the endometrium damaged during transfer? are vaginal or cervical bacteria introduced?) and the place of deposition of the embryo.

Table 2. Pregnancy rates after multiple embryo transfer

Embryos transferred	Number of transfers	Number of pregnancies (% of transfers)	Multiple pregnancies (% of pregnancies)
1 (natural cycle)	250	38 (15.0)	–
1 (stimulated cycle)	795	105 (13.2)	1 (monozygotic)
2	563	140 (24.8)	22 (16%)
3	242	71 (29.3)	7 (10%)
4 or more	133	41 (31.0)	17 (41%)

The data in this table are based on Kerin et al.[30]; Muasher et al.[43]; Edwards et al.[17]; Craft et al.[12]; Lopata[34].

The embryonic factor might mean that only a certain number of the embryos produced by in vitro fertilization are able to implant. An important factor in uterine receptivity might be the maternal age. Edwards and Steptoe[17] found an increased incidence of abortion and a decreasing incidence of implantation with increasing age. Many patients who enroll in IVF programs are in their thirties, if not older. This might influence the success-rates negatively. A correct evaluation of success after in vitro fertilization can only be done by comparing in vitro with natural reproductive efficiency. The latter has been estimated by calculation and by direct observation of biological data. Leridon[32] calculated that the expectancy of a term delivery per cycle of exposure was 31%. The pregnancy loss within two weeks after conception was estimated to be 58%. Edmonds et al.[15] found similar high rates of early pregnancy loss via a sensitive radioimmunoassay for β-HCG. It is not known how this loss is caused. If embryonic factors such as chromosomal abnormalities, incompatible with implantation or further embryonic development, are involved, it may be inferred that under in vitro conditions also, a portion of the developed embryos will possess these abnormalities. It may well be that the first cleavage stages progress normally, but that the embryo becomes arrested at a later stage, i.e. after embryo transfer. In a study by Angell et al.[2] chromosomal abnormalities in two out of three investigated human preimplantation embryos were described. This study demonstrates that chromosomal aberrations are at least of concern in the analysis of embryonic loss.

There are other factors which might influence implantation of embryos after transfer. The in vitro fertilization procedure itself might be a negative factor. In an analysis of the benefits and risks of multiple transfer, Speirs et al.[51] suggested that at present the quality of the embryos after IVF is rather low. This can be concluded from the low twinning rates after the transfer of two or more embryos (table 2). If the uterine receptivity was the limiting step, many more twins should be expected because once the uterus is receptive for an embryo it should accept them all. Speirs et al.[51] estimate that only 10–20% of the embryos would survive in a receptive uterus.

Early maternal recognition

Most discussions on embryonic wastage after IVF/ET do not mention the possibility that a fertilized ovum is recognized at a very early stage by the maternal organism. So the endometrium, or the maternal organism as a whole, is prepared for pregnancy. If this very early pregnancy recognition exists, embryos transferred after in vitro fertilization would experience a disadvantage compared with embryos arriving in the uterus after natural conception. This fact might help to explain why the embryonic wastage is still high in comparison with the natural cycle. The aforementioned figure of 12.9% sur-

vivors (table 2) is certainly less than the estimated 30% in the natural cycle.

In this context, the discovery of an immunosuppressive early pregnancy factor (E.P.F.) must be mentioned. E.P.F. was detected in sheep and mouse within 24 h of the presumed fertilization[10,42]. E.P.F. activity has also been detected in human serum[57] and in the culture fluid of human embryos[49].

The discovery of an embryo-derived blood platelet activating factor in blood during the preimplantation period[45] is another example of the interaction between embryo and maternal organism. Such factors may become an important means to monitor the viability of embryos after in vitro fertilization. If their existence is biochemically confirmed, they might provide us with important clues on natural and in vitro conception.

Acknowledgments. The authors thank A. Roatti for skillful technical assistance in the in vitro fertilization program. This work was supported by grant no. 180 Hoffmann-La Roche Arbeitsgemeinschaft and grant no. 3.916-0.82 of the Swiss National Science Foundation.

1 Aitken, R.J., Best, F.S.M., Richardson, D.W., Djahanbakch, O., Mortimer, D., Templeton, A.A., and Lees, M.M., An analysis of sperm function in cases of unexplained infertility: conventional criteria, movement characteristics and fertilizing capacity. Fert. Steril. *38* (1982) 212–221.

2 Angell, R.R., Aitken, R.J., Van Look, P.F.A., Lumsden, M.A., and Templeton, A.A., Chromosome abnormalities in human embryos after in vitro fertilization. Nature *303* (1983) 336–338.

3 Austin, C.R., The 'capacitation' of the mammalian sperm. Nature *170* (1952) 326.

4 Austin, C.R., Membrane fusion events after fertilization. J. Reprod. Fert. *44* (1975) 155–166.

5 Bedford, J.M., Ultrastructural changes in the spermhead during fertilization in the rabbit. Am. J. Anat. *133* (1968) 213–254.

6 Bedford, J.M., Mechanisms involved in penetration of spermatozoa through the vestments of the mammalian egg, in: Physiology and Genetics of Reproduction, Part B, p. 55. Eds E.M. Coutinho and F. Fuchs. Plenum, New York 1974.

7 Bedford, J.M., and Cooper, G.W., Membrane fusion events in the fertilization of vertebrate eggs, in: Cell Surface Reviews, vol. 5, pp. 65–125. Elsevier, Amsterdam 1978.

8 Blerkom, J., Henry, G., and Parreco, R., Preimplantation human embryonic development from polypronuclear eggs after in vitro fertilization. Fert. Steril. *91* (1984) 686–696.

9 Chang, M.C., The fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature *168* (1951) 697.

10 Clarke, F.M., Morton, H., Rolfe B.E., and Clunie, G.J.A., Partial characterization of early pregnancy factor in the sheep. J. Reprod. Immun. *2* (1980) 151–162.

11 Cohen, J., Edwards, R.G., Fehilly, C., Fishel, S., Henstt, J., Purdy, J., Rowland, G., Steptoe, P., and Webster, J., In vitro fertilization: a treatment for male infertility. Fert. Steril., in press (1985).

12 Craft, I., Porter, R., Green, S., Tucke, M., Smith, B., Twigg, H., Attuja, K., and Whittingham, D., Success of fertility, embryo number and in vitro fertilization. Lancet *1* (1984) 732.

13 Cummins, J.M., and Yanagimachi, R., Sperm egg ratio's and the site of the acrosome reaction during in vivo fertilization in the hamster. Gamete Res. *5* (1982) 239–256.

14 Davis, B.K., Byrne, R., and Bedigian, K., Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during in vitro incubation of rat sperm cells. Proc. natn. Acad. Sci. *77* (1980) 1546–1550.

15 Edmonds, D.K., Lindsay, K.S., Miller, J.F., Williamson, E., and Wood, P.J., Early embryonic mortality in women. Fert. Steril. *38* (1982) 447–452.

16 Edwards, R.G., and Purdy, J., Fertilization and cleavage in vitro of preovulatory human oocytes. Nature *227* (1970) 1307.

17 Edwards, R.G., and Steptoe, P.C., Current status of in vitro fertilization and implantation of human embryo's. Lancet *2* (1983) 1265–1269.

18 Epel, D., Fertilization. Endeavour [n.s.] *4* (1980) 26–47.

19 Fishel, S.B., Edwards, R.G., and Purdy, J.M., Births after a prolonged delay between oocyte recovery and fertilization in vitro. Gamete Res. *9* (1984) 175–181.

20 Florman, H.M., and Storey, B.T., Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization in vitro. Devl Biol. *91* (1982) 121–130.

21 Fowler, R.E., Edwards, R.G., Wallers, D.E., Chan, S.T.H., and Steptoe, P.C., Stereoidogenesis in preovulatory follicles of patients given human menopausal and chorionic gonadotropins as judged by the radioimmunoassay of steroids in follicular fluid. J. Endocr. *77* (1978) 161–169.

22 Fraser, L.R., p-Aminobenzamidine, an acrosin inhibitor, inhibits mouse sperm penetration of the zona pellucida, but not the acrosome reaction. J. Reprod. Fert. *65* (1982) 185–194.

23 Gwatkin, R.B.L., Williams, D.T., Harmann, J.F., and Kniazuk, M., The zona reaction of hamster and mouse eggs: Production in vitro by a trypsin like protease from cortical granules. J. Reprod. Fert. *32* (1973) 259–265.

24 Harrison, R.A.P., The acrosome, its hydrolases and egg penetration, in: The Sperm Cell, pp. 259–273. Ed. J. André. Nijhoff, The Hague 1983.

25 Johnstone, I., Lopata, A., Speirs, A., Hoult, I., Kellow, G., and DuPlessis, Y., In vitro fertilization: a challenge of the eighties. Fert. Steril. *36* (1981) 699–706.

26 Jones, H.W., Acosta, A.A., Garcia, J.E., Sandow, B.A., and Veeck, L., On the transfer of conceptuses from oocytes fertilized in vitro. Fert. Steril. *39* (1983) 241–243.

27 Juetten, J., and Bavister, B.D., Effects of egg aging on in vitro fertilization and first cleavage division in the hamster. Gamete Res. *8* (1983) 219–230.

28 Kanwar, K.C., Yanagimachi, R., and Lopata, A., Effects of human seminal plasma on fertilizing capacity of human spermatozoa. Fert. Steril. *31* (1979) 321–325.

29 Kemeter, P., and Feichtinger, W., Pregnancy following in vitro fertilization and embryo transfer using pure human serum as culture and transfer medium. Fert. Steril. *41* (1984) 936–937.

30 Kerin, J.F., Quinn, P.J., Kirby, C., Seamark, R.F., Warnes, G.M., Jeffrey, R., Mathews, C.D., and Cox, C.W., Incidence of multiple pregnancy after in vitro fertilization and embryo transfer. Lancet (Sept. 1983) 531–540.

31 Langlais, J., Zolliger, M., Plante, L., Chapdelaine, A., Bleau, G., and Roberts, K.D., Localization of cholesteryl sulphate in human spermatozoa in support of a hypothesis for the mechanism of capacitation. Proc. natn. Acad. Sci. USA *75* (1981) 7266–7270.

32 Leridon, H., Human fertility. The basic components. University of Chicago Press, Chicago 1977.

33 Leung, P.C.S., Gronow, M.J., Kellow, G., Lopata, A., Speirs, A.L., Mc Bain, J., DuPlessis, Y.P., and Johnston, I., Serum supplement in human in vitro fertilization and embryo development. Fert. Steril. *41* (1984) 36–50.

34 Lopata, A., Concepts in vitro fertilization. Fert. Steril. *40* (1983) 289–301.

35 Lopata, A., Jones, G.M., Nayudu, P., Gook, D., DuPlessis, Y., Bourne, H., and Leuran, D., The potential of the human egg to produce viable embryo's and pregnancies. J. In Vitro Fert. Embryo Transfer *1* (1984) 122.

36 Lopata, A., Sathananthan, H., Mc Bain, J., Johnstone, W.I.H., and Speirs, A., The ultrastructure of the preovulatory human egg fertilized in vitro. Fert. Steril *33* (1980) 12.

37 Lopata, A., and Wood, C., In vitro fertilization and embryo transfer: its clinical application. Oxford Rev. reprod. Biol. *4* (1982) 148–194.

38 McRorie, R.A., and Williams, W.L., Biochemistry of mammalian fertilization. A. Rev. Biochem. *43* (1974) 777–803.

39 Meizel, S., and Working, P.K., Further evidence suggesting the hormonal stimulation of hamster sperm acrosome reactions by catecholamines in vitro. Biol. Reprod. *22* (1980) 211–216.

40 Mortimer, D., Selectivity of spermtransport in the female genital tract, in: Spermatozoa, Antibodies and Infertility, pp. 37–53. Eds J. Cohen and W.F. Hendry. Blackwell, Oxford 1978.

41 Mortimer, D., Leslie, E.E., Kelly, R.W., and Templeton, A.A., Morphological selection of human spermatozoa in vivo and in vitro. J. Reprod. Fert. *64* (1982) 391–399.

42 Morton, H., Rolfe, B., McMull, L., and Clunie, G.J.A., Early pregnancy factor tissues involved in its production in the mouse. J. Reprod. Immun. *3* (1980) 73–82.

43 Muasher, S.J., Wilkes, C., Garcia, J.E., Rosenwaks, Z., and Jones, H.W., Benefits and risks of multiple embryo transfer with in vitro fertilization. Lancet *1* (1984) 570.

44 Nayudu, P.L., Lopata, A., Leung, P.C.S., and Johnston, W.I.H., Current problems in human in vitro fertilization and embryo implantation. J. exp. Zool. *228* (1983) 203–213.
45 O'Neill, C., and Saunders, D.M., Assessment of embryo quality. Lancet *2* (1984) 1035.
46 Quinn, P.J., Warnes, G.M., Kerin, J.F., and Kirby, C., Culture factors in relation to the success of human in vitro fertilization and embryo transfer. Fert. Steril. *41* (1984) 202–209.
47 Quinn, P.J., Warnes, G.M., Kerin, J.F., and Kirby, C., Culture factors affecting the success rate of in vitro fertilization and embryo transfer. Ann. N.Y. Acad. Sci., in press (1985).
48 Sathananthan, H., and Trounson, A.O., Ultrastructural observations on cortical granules in human follicular oocytes cultured in vitro. Gamete Res. *5* (1982) 191–198.
49 Smart, Y.C., Cripps, A.W., Clancy, R.L., Lopata, A., and Shutt, D.A., Detection of an immunosuppressive factor in human preimplantation embryo culture. Med. J. Aust. *1* (1981) 78–79.
50 Soupart, P., and Strong, P.A., Ultrastructural observations on polyspermic penetration of zona pellucida free human oocytes inseminated in vitro. Fert. Steril. *26* (1975) 523–536.
51 Speirs, A.L., Lopata, A., Gronow, M., Kellow, G., and Johnstone, I., Analysis of the benefits and risks of multiple embryo transfer. Fert. Steril. *39* (1983) 468–471.
52 Stambaugh, R., Enzymatic and morphologial events in mammalian fertilization. Gamete Res. *1* (1978) 65–85.
53 Stambaugh, R., and Buckley, J., Identification and subcellular localization of the enzymes effecting penetration of the zona pellucida by rabbit spermatozoa. J. Reprod. Fert. *19* (1969) 423–432.
54 Talbot, P., and Chacon, R.S., Ultrastructural observations on binding and membrane fusion between human sperm and zona pellucida free hamster oocytes. Fert. steril. *37* (1982) 240–249.
55 Tea, N.T., Jondet, M., and Scholler, R., Procédé d'isolement des spermatozoïdes mobiles du sperme humain par la méthode de 'migration-sedimentation'. Path. Biol. *31* (1983) 688–690.
56 Testart, J., Lassalle, B., Frydmann, R., and Belaisch, J., A study of factors affecting the success of human fertilization in vitro II. Biol. Reprod. *20* (1983) 425–431.
57 Thomson, A.W., Milton, J.F., Camphell, D.M., and Home, C.W., Rosette inhibition levels during early human gestation. J. Reprod. Immun. *2* (1980) 263–268.
58 Trotnow, S., Al-Hasani, S., Kniewald, T., Becher, H., and Hunlich, T., Transfer of human embryos at early cleavage stages, in: Fertilization of the Human Egg in vitro, pp. 283–306. Eds Beier and Lindner. Springer-Verlag, Berlin 1983.
59 Trounson, A., In vitro fertilization. Curr. Topics exp. Endocr. *5* (1983) 43–75.
60 Trounson, A.O., Mohr, C.R., Wood, C., and Leeton, J.F., Effect of delayed insemination on in vitro fertilization, culture and transfer of human embryo's. J. Reprod. Fert. *64* (1982) 285–294.
61 Veeck, L.L., Wortham, J.W.E., Withmyer, J., Sandow, B.A., Acosta, A.A., Garcia, J.E., Jones, G.J., and Jones, H.W., Maturation and fertilization morphologically immature human oocytes in a program of in vitro fertilization. Fert. Steril. *39* (1983) 594–602.
62 Wolf, D.P., The mammalian block to polyspermy, in: Fertilization and embryonic development in vitro, pp. 183–197. Eds C. Mastroianni and J.B., Biggers. Plenum, New York 1981.
63 Wolf, D.P., Inoue, M., and Stark, R.A., Penetration of zona-free mouse ova. Biol. Reprod. *15* (1976) 213–221.
64 Wortham, J.W.E., Veeck, L.L., Witmyer, J., Sandow, B.A., and Jones, H.W., Vital initiation of pregnancy (VIP) using human menopausal gonadotropin and human chorionic gonadotropin ovulation induction: Phase II, 1981. Fert. Steril. *40* (1983) 170–177.
65 Yanagimachi, R., Mechanisms of fertilization in mammals, in: Fertilization and Embryonic Development in vitro, pp. 81–182. Eds L. Mastroianni and J.D. Biggers. Plenum, New York 1981.
66 Yanagimachi, R., Zona-free hamster eggs: their use in assessing fertilizing capacity and examing chromosomes of human spermatozoa. Gamete Res. *10* (1984) 187–232.
67 Yanagimachi, R., and Noda, Y.D., Electron microscopic studies of sperm incorporation into the golden hamster egg. Am. J. Anat. *128* (1970) 429–462.
68 Yanagimachi, R., and Philips, D.M., The status of acrosomal caps of hamster spermatozoa immediately before fertilization in vivo. Gamete Res. *9* (1984) 1–19.
69 Yanagimachi, R., and Usui, N., Calcium dependence of the acrosome reaction and activation of guinea-pig spermatozoa. Exp. Cell Res. *819* (1974) 161–174.
70 Yovich, J.L., Kay, D., Stanger, J.D., and Boetcher, B., In vitro fertilization of oocytes from women with serum antisperm antibodies. Lancet *8373* (Feb. 1984) 369–370.
71 Yovich, J.L., and Stanger, J.D., The limitations of in vitro fertilization from males with severe oligospermia and abnormal sperm morphology. J. in Vitro Fert. Embryo Transfer *1* (1984) 172–179.

0014-4754/85/121496-07$1.50 + 0.20/0

Ovarian stimulation with clomiphene and/or human menopausal gonadotropin (HMG) for in vitro fertilization (IVF) and embryo transfer (ET)

by P. Kemeter and W. Feichtinger

Institute for Reproductive Endocrinology and In Vitro Fertilization, Trauttmansdorffgasse 3A, A–1130 Vienna (Austria)
Key words. In vitro fertilization; ovarian stimulation; clomiphene; human menopausal gonadotropin; follicle; oocyte.

The pregnancy rate in IVF-ET programs appears to be directly proportional to the number of concepti transferred[5]. Therefore, an essential prerequesite for ultimate success is the recruitment and maturation of an adequate number of oocytes. To accomplish this aforementioned goal three principal stimulation programs have evolved. 1. Clomiphene only, 2. Clomiphene plus HMG in varying modifications and 3. HMG only.

At its inception our program admitted patients with unstimulated normal menstrual cycles. Due to an unacceptably low success rate we changed to ovarian stimulation with clomiphene shortly thereafter.

As soon as the Australian group of The Royal Women's Hospital in Melbourne had reported on increasing pregnancy rates using HMG in addition to clomiphene[11] we introduced the clomiphene-HMG protocol of McBain et al.[12]. Finally, we stimulated with HMG only according to the Norfolk protocol[7] primarily in those patients who did not respond properly to clomiphene or clomiphene-HMG.

The purpose of this paper is to present our experience with the above mentioned stimulation protocols in regard to hormone parameters as well as to fertilization and pregnancy outcome.

Number of follicles, oocytes and fertilizations

McBain and co-workers found a disparity between the number of follicles seen at laparoscopy and the greater number observed ultrasonically earlier in the cycle when