Recent advances in clinical aspects of in vitro fertilisation

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While early success rates using in vitro fertilisation ranged from 10% to 15%, improved technology has more than doubled that rate, owing to the availability of recombinant follicle-stimulating hormone, improved ovarian stimulation protocols, and assisted fertilisation technology. This latter technology has already revolutionised the management of male-factor infertility. The future goal is to further increase the overall pregnancy rate and to reduce the risk of multiple gestation by using pre-implantation genetic diagnosis and blastocyst transfer.

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In most western countries, 1% of children are conceived by some form of assisted reproductive technology (ART).[1] Since the birth of Louise Brown in 1978, in vitro fertilisation (IVF) has become the therapeutic mainstay for female infertility. In 1992, assisted insemination by using intracytoplasmic sperm injection (ICSI), a scion of IVF technology, heralded a new era in the treatment of male-factor infertility. There were rapid developments on many fronts in ART in the closing years of the past century.

Recombinant follicle-stimulating hormone

Exogenous gonadotrophins have been widely used in the past 30 years to induce ovulation and their use has undergone considerable changes, especially with regard to the purification process. Follicle-stimulating hormone (FSH) as human menopausal gonadotrophin (HMG) is extracted from urine and has a purity of only 5%; there is considerable batch-to-batch variance in its activity. The production of HMG is difficult and subject to the vagaries of human supply. A recent increase in the worldwide demand for gonadotrophins has necessitated major urine collection programmes.

By the mid-1990s, it was clear to suppliers of HMG, that the future of gonadotrophin supply could not rely on urinary sources alone. Follicle-stimulating hormone has recently been manufactured by using recombinant DNA technology. The hormone is produced by a Chinese hamster ovary cell line that has been transfected with the genes encoding FSH.[2] Sources of recombinant FSH (rFSH) allow the large-scale production of a preparation that is almost totally (99%) pure and which can be administered subcutaneously or intramuscularly. Unlike urinary human FSH, rFSH contains no luteinizing hormone (LH) and has excellent batch-to-batch consistency. The terminal half-lives of the two preparations are similar: urinary FSH, 36 hours and rFSH, 40 hours.[3]

Recombinant FSH and natural FSH differ slightly in their carbohydrate moeities.[4] In addition, minute amounts of contamination derived from host cells might be present in the recombinant preparations. However, antibody development against FSH and host-cell proteins has never been seen in rFSH-treated patients. Studies using rFSH have recently shown improved fertilisation rates.[5] Furthermore, the duration of ovarian stimulation is shorter, and fewer ampoules of rFSH are needed. While rFSH will ultimately replace HMG, the latter is a cheaper product of proven efficacy and will still have a place in the management of infertility, especially in developing countries.

Recombinant luteinizing hormone

In the early 1970s, LH that had been extracted from human pituitary glands had been shown to be able to trigger ovulation. Deterrents to the development of this therapeutic modality, however, included its high

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cost and the possible transmission of communicable diseases such as Creutzfeld-Jakob disease or infection with human immnuodeficiency virus. On the other had, the use of urine-derived human chorionic gonadotrophin (HCG) as an LH surrogate entailed the same logistic problems that beset the commercial production of HMG.

Luteinizing hormone has been recently produced from genetically engineered Chinese hamster ovary cells. The recombinant LH (rLH) product is pure and free of viral contamination. Furthermore, its specific immuno-activity, bioactivity, volume distribution, and total clearance and elimination rates are similar to those of human LH.[6] Given intravenously, rLH has an initial half-life of 1 hour and a terminal half-life of 11 hours; the corresponding values for human LH are 0.6 hours and 10 hours, respectively.[6]

Currently, rLH is undergoing phase III trials and will not be available commercially for at least another year. Its main clinical application is expected to be in the treatment of group 1 anovulation, as classified by the World Health Organization,[7] or hypogonadotropic hypogonadism—a rare condition characterised by reduced hypothalamic or pituitary activity, which results in abnormally low FSH and LH levels. The recommended daily dose of rLH is 75 IU by subcutaneous administration; a minority of patients may need up to 225 IU/d. Drug treatment has been shown to be well tolerated and not immunogenic.[8]

Gonadotrophin-releasing hormone antagonists

The gonadotrophin-releasing hormone (GnRH) agonists were introduced in the 1990s to suppress the production of endogenous pituitary gonadotrophins, to achieve the full control of follicular recruitment and development by using exogenously administered gonadotrophins. Given in a pulsatile fashion, GnRH agonists can stimulate LH production. In contrast, when GnRH agonists are given in a non-pulsatile fashion (eg subcutaneously, by depot, or by daily injection), they have an initial flare-up effect, causing a brief surge of pituitary gonadotrophins and depletion and downregulation of LH receptors in the pituitary gland. Because of the initial flare-up effect, it takes 7 to 8 days for GnRH agonists to achieve a suppressive effect on the pituitary gonadotrophins. Thus, patients need more aggressive stimulation with exogenously administered FSH, which means increased drug costs.

The GnRH antagonists are far more complex than the GnRH agonists; the former group of proteins have modifications in their molecular structure at amino acid positions 1, 2, 3, 6, 8, and 10. They have an entirely different mechanism of action, in that they bind immediately to the GnRH receptors, but do not elicit a biological response. Their main effect is to prevent a premature LH surge, which can be overruled or reversed by the concomitant administration of GnRH. Ceasing treatment with GnRH antagonists leads to normalisation within 24 hours. In contrast, GnRH agonists act for much longer. The mechanism of action of GnRH antagonists is dependent on the equilibrium between endogenous GnRH and the applied antagonist.[9] Consequently, the effect of GnRH antagonists is highly dose-dependent, unlike the GnRH agonists.

The first-generation GnRH antagonists were not clinically useful because of the complication of histamine release. The third-generation drugs, however, induce a low level of histamine release and can thus be used in clinical practice. The use of GnRH antagonists given subcutaneously has been shown to slightly decrease the pregnancy rate and, more importantly, to decrease the rate of ovarian hyperstimulation syndrome from 5% to 2%.[10,11] They are given around day 5 to day 7 of ovarian stimulation, depending on the dose regimen, which may be single- or dual-dosage (French protocol)[12] or multiple-dosage (Lübeck protocol).[13] The incidence of a surge in the LH level is less than 2%; luteal support remains mandatory.[14] Ovulation induction is possible by using GnRH agonists or native GnRH itself under antagonistic treatment. This regimen could help reduce the incidence of an early onset of ovarian hyperstimulation syndrome.[15]

Because endogenous pituitary gonadotrophins are not suppressed in the early follicular phase, FSH treatment time is shorter and fewer ampoules are needed. Using drugs that cause a milder stimulation of gonadotrophin release such as clomifene (clomiphene) citrate may be a cheaper and safer way of achieving ovarian stimulation.[16] It is hoped that, given time and experience, the pregnancy rate achieved by using GnRH antagonists will catch up with that achieved by using GnRH agonists.

Blastocyst culture and transfer

It is an accepted practice worldwide to transfer embryos on day 2 (around the four-cell stage) or on day 3 (around the eight-cell stage) of development. Such cleavage-stage embryos in vivo reside in the fallopian tube and not the uterus. In a prospective, controlled study of 790 patients undergoing IVF, Gardner et al[16] showed that the pregnancy rate per embryo transfer

was 35.9% in the control group, in which a mean of 3.0 cleavage-stage embryos were transferred on day 3. In contrast, the pregnancy rate per embryo transfer was 43.8% in the group in which a mean of 2.5 blastocyst-stage embryos were transferred on day 5.[16] However, in a prospective controlled study of 233 patients, Scholtes and Zeilmaker[17] found no difference in the overall pregnancy rate between the groups that had day-3 and day-5 transfers (26% versus 25%, respectively). Scholtes and Zeilmaker[17] used single-culture medium throughout the blastocyst culture period, whereas Gardner et al[16] used sequential two-step culture media, which enable the embryo to develop to the blastocyst stage without losing developmental competence.

Extended culture also has other advantages. It enables the embryo to be synchronised with the female genital tract, which helps improve implantation. Hence, fewer embryos are needed for transfer, thereby decreasing the incidence of high-order multiple pregnancies. Furthermore, embryologists are better able to assess embryo development and viability at the blastocyst stage. The additional 2 to 3 days' culture allows pre-implantation genetic diagnosis (PGD) to be performed between biopsy on day 3 and blastocyst transfer on day 5 or 6. The generation of blastocysts thereby allows a trophectodermal biopsy examination to be conducted; this test is the earliest form of genetic diagnosis of non-embryonic material.

Intracytoplasmic sperm injection

In the mid-1980s, it became clear that conventional IVF had limitations in the alleviation of long-standing male infertility. Couples were not accepted for IVF if the semen parameters were overly impaired, and if a substantial number of treatment cycles did not result in embryo transfer. In 1992, researchers from the Brussels Free University, Belgium, reported the first pregnancies and births resulting from the replacement of embryos that were generated by ICSI.[18] Since then, ICSI has been incorporated into ART for couples with severe male-factor infertility.

Severe teratospermia

In a 20-patient study,[19] conventional IVF and ICSI were performed using severe teratospermic (<4%, Kruger strict criteria)[20] specimens to fertilise sibling oocytes. Intracytoplasmic sperm injection achieved a fertilisation rate of 66%, compared with 48% for IVF.[19]

Complete asthenospermia

The major technical problem associated with using immotile sperm for ICSI is differentiating between live and dead sperm. In most cases of complete asthenospermia, less than 50% are viable. The standard techniques of vital dye staining to assess the integrity of the sperm membrane and, by inference, sperm viability, all result in the death of the sperm.

The hypo-osmotic swelling test (HOST) has been used to select viable sperm for ICSI in men with complete asthenospermia. Sperm with a functional membrane that can transport fluid will undergo swelling of the cytoplasmic space, thereby causing the sperm tail fibres to curve. These changes are visualised easily using light microscopy. In this way, viable sperm can be selected from a population of completely immotile sperm for ICSI. The fertilisation and cleavage rates achieved by ICSI using sperm selected by HOST have been shown to be 43% and 39%, respectively; when randomly selected sperm were used, the rates were 26% and 23%, respectively.[21]

Cryptozoospermia

Cryptozoospermia are often found in the pellet of the centrifuged ejaculate of obstructive and non-obstructive azoospermic men and can be used for ICSI/IVF. Embryo development shows no difference with regard to sperm origin, be it ejaculate, epididymis, or testis.[22,23]

Non–male-factor infertility

The question of whether the indications for ICSI should be extended to patients with non-male infertility—that is tubal, idiopathic, and borderline male-factor infertility—awaits the outcome of randomised controlled trials that compare IVF and ICSI, especially with regard to the long-term health of the children. The results of a study of 116 patients with tubal factor infertility who were divided randomly between two groups, showed that ICSI did not lead to a higher pregnancy rate than conventional IVF.[24]

Genetic disorders

Genetic disorders may sometimes underlie severe oligospermia and azoospermia in males undergoing ICSI-assisted reproduction. The application of ICSI technology to the treatment of male infertility and, in particular, severe male-factor infertility may result in the transmission of genetic or chromosomal abnormalities to the offspring.[25] The incidence of 46 major congenital malformations among 1966 children born alive was found to be 2.3%; the rates were 2.1% among singletons, 2.7% among twins, and 2.0% among triplets.[26] These rates are similar to the those found in national registries of ART surveys.[26] The issue of using immature male germ cells, such as round

spermatids, in ICSI remains unresolved. The fertilisation and pregnancy rates, however, generally remain far below those obtained with mature sperm and elongated spermatids.[27]

Bonduelle et al[28] found abnormal foetal karyotypes in 15 of 690 amniocenteses and 13 of 392 chorionic villi samplings. Among these 1082 samples, there were 18 (1.7%) de novo chromosomal aberrations: half (9/1082; 0.8%) of which were X-chromosome aberrations; the other half (9/1082; 0.8%) were autosomal aberrations, such as trisomies and structural aberrations.[28] These frequencies of sex-chromosomal aberrations are significantly higher than those observed in non-ICSI conceived newborns (0.14% to 0.19%).[29,30] In all the cases of de novo chromosomal aberrations that were observed by Bonduelle at al,[28] however, the sperm had come from men with severe oligoasthenoteratozoospermic defects (concentration, 0.1-4.6 million/mL; eumorphs, 0%-40%; progressive motility, 0%-18%).

A gene called *DAZ* (deleted in azoospermia) is the leading, but not exclusive, candidate gene in the *AZF* (azoospermia factor) locus for influencing spermatogenesis.[31] The *DAZ* gene is consistently deleted in men with abnormal spermatogenesis, at approximately 14% in azoospermic men and 10% in severely oligospermic men.[32] Microdeletion in the variable region of Yq in the *AZF* locus can lead to a 'Sertoli cell–only' phenotype, maturation arrest, and severe oligospermia.[32] The microdeletion of *AZF* and gonadal mosaicism are transmittable genetic anomalies that are common among infertile men with a karyotype of 46, XY. Another genetically transmittable disorder that affects male fertility is cystic fibrosis, which is also one of the most common genetic disorders among Caucasians of European descent. This autosomal recessive disease, in which patients have a congenital bilateral absence of the vas deferens, is found in 0.5% of male autopsies.[33]

When ICSI is offered to couples for the treatment of male-factor infertility, it is essential for them to have an understanding of the genetic implications of the procedure. When possible, they should be offered genetic screening of both the male partner and the ICSI-generated embryos prior to their replacement.

Prognostic assessment of ovarian reserve

A decline in oocyte quality and quantity rather than diminished endometrial receptivity is the major cause of reduced reproductive performance in older women. Since the introduction of IVF, considerable effort has been expended on investigating the ovarian reserve of this group of patients.

Measuring basal levels of luteinizing hormone

In 1985, Stanger and Yovich[34] reported a study of 60 IVF patients, nine of whom had elevated basal LH levels, which was defined as an LH concentration of more than one standard deviation. These patients had a significantly reduced rate of oocyte fertilisation. Fifty-nine patients subsequently underwent embryo transfer and of the 10 clinical pregnancies, none occurred in those with elevated LH values.

Other investigators have used different definitions of LH hypersecretion: Regan et al[35] have used an absolute value of 10 mIU/mL (10 IU/L) while others have used a ratio of LH to FSH of 1.5.[36]

Measuring day-3 levels of follicle-stimulating hormone

In a retrospective study of 758 IVF patients, Muasher et al[37] found that pregnancy rates decreased markedly as day-3 basal FSH levels rose. Ongoing pregnancy rates were highest in women whose FSH levels were less than 15 mIU/mL, and rates fell to less than 5% in those whose basal FSH levels were more than 25 mIU/mL.

Measuring day-3 levels of both oestradiol and follicle-stimulating hormone

Buyalos et al[38] have shown that using the day-3 levels of serum oestradiol (E_2) and FSH in combination is a better indicator of diminished ovarian reserve (DOR) than using the level of FSH alone, when the basal E_2 and FSH levels are ≤80 pg/mL and ≤13 mIU/mL, respectively.

Clomifene citrate challenge test

In 1987, Navot et al[39] described the use of the clomifene citrate challenge test, in which oral clomifene 100 mg/d was given from days 5 to 9 to assess ovarian reserve in 51 women aged 35 years or older. They found that although 18 of the women had a normal baseline FSH level, 18 had an exaggerated FSH response on day 10 (≥26 mIU/mL: more than two standard deviations above control values). These women were regarded as having DOR (pregnancy rate, 6%), compared with women with an FSH level of 11.5 mIU/mL, who had adequate ovarian reserve (AOR) and a pregnancy rate of 42%. The premise of this test is that in women with AOR, the overall metabolic activity of the developing follicles should be able to overcome the impact of clomifene on the hypothalamic-pituitary axis and suppress FSH levels to the normal range by day 10.

The test can detect DOR patients who might not be identified by measuring the basal FSH alone.

Patients with AOR who have a normal basal FSH level (<12 mIU/mL) have low intercycle variations FSH concentration, whereas those with DOR have an elevated basal FSH and a higher degree of variation. Loumaye et al[40] redefined an abnormal result of the clomifene challenge test, by adding the day-3 and day-10 FSH values. In their series of 114 patients, a threshold effect was evident, as pregnancy rates remained equivalent until the summed FSH concentrations exceeded 26 mIU/mL; the rates then became 0%. The prediction value of a woman with an abnormal result subsequently failing to become pregnant was 100%.

Measuring inhibin B levels

The pituitary secretion of FSH is co-regulated by oestrogen and two dimeric polypeptides, inhibin A and inhibin B. Both inhibins are produced by the granulosa cells of the ovarian follicle, with inhibin B being secreted predominantly in the follicular phase. Inhibin A is secreted predominantly in the luteal phase and by extragonadal tissues; it is less useful as a prognostic indicator of an ovarian reserve than inhibin B. Research on the regulatory role of the inhibins in humans has been hampered by the lack of highly sensitive and specific assays for these compounds.

By measuring the level of inhibin B on day 3 and day 10 in 24 subfertile women undergoing the clomifene challenge test, Hofmann et al[41] demonstrated that all women had significantly higher inhibin B levels on day 10 than on day 3. In addition, women with AOR had higher inhibin B levels on both day 3 and 10 (94 [standard deviation, 37] pg/mL and 239 [132] pg/mL, respectively) when compared with the women who had DOR (day-3, 59 [36] pg/mL; day-10, 136 [74] pg/mL). In all women with DOR, the day-3 and day-10 FSH levels were also abnormally low—that is, 28 (18) mIU/mL and 42 (19) mIU/mL, respectively.

Premature follicular luteinization

Younis and et al[42] showed that 19 of 31 IVF patients with unexplained infertility had premature follicular luteinization, which was defined as a ratio of progesterone to E_2 of 1 on the day of HCG administration. The mean age, duration of infertility, and day-3 FSH levels between the study and control groups were similar. The total dose of HMG and duration of administration were also similar. The clinical pregnancy rates after four cycles of treatment in the study and control groups were 15.8% and 41.7%, respectively. The study group also showed higher basal day-3 E_2 levels, diminished maximum E_2 levels on the day of HCG administration, and fewer mature follicles. These results may be explained by an elevation of day-10 progesterone level, as shown by Hofmann et al,[43] in a study of patients with DOR. The phenomenon of premature follicular luteinization has also been observed by other investigators in IVF cycles in which the women have been given GnRH agonist–induced downregulation.[14,44] These studies found that premature follicular luteinization was associated with decreased pregnancy rates.

In summary, ovarian primordial follicles diminish in quality and number with a woman's age, which results in a diminished number of follicular granulosa cells and inhibin production. These changes are reflected by a diminished production of E_2 in the follicular phase and a corresponding elevation of FSH, LH, progesterone, and the progesterone to E_2 ratio. Subtle endocrinopathies in patients with DOR can sometimes be detected only by using the clomifene challenge test or HMG stimulation.

Pre-implantation genetic diagnosis

Pre-implantation genetic diagnosis can be performed before or after fertilisation. Performing a polar body biopsy provides an option for couples who are at risk of conceiving a genetically abnormal foetus to avoid the birth of an affected child without the need for a selective abortion of an affected foetus following PGD. The first polar body is extruded from a metaphase II oocyte at the time of ovulation and the second polar body is extruded after fertilisation by a spermatozoon. These two steps can be performed separately at stages—that is, at ovulation and after insemination, or together after insemination. These polar bodies have no biological significance in embryonic development and studies have shown no detrimental effect on the rates of post-biopsy fertilisation and implantation, blastocyst formation, or pregnancy.

Blastocyst biopsy followed by PGD allows the genetic analysis of a large number of trophectodermal cells. Because trophectodermal cells develop into the placenta and membranes, injury to the inner cell mass, which develops into the embryo, is avoided. In addition, because the blastocyst comprises more than 200 cells, cells can be analysed in duplicate to confirm the diagnosis.

Polymerase chain reaction (PCR) analysis is instrumental in the diagnosis of single-gene defects, including cystic fibrosis, thalassaemia, Tay-Sachs disease,

haemophilia A, haemophilia B, retinitis pigmentosa, sickle cell disease, Alport's syndrome, α1-antitrypsin deficiency, Gaucher's disease, and long-chain acyl-Co A dehydrogenase deficiency. By using this technique to analyse the first polar body, first meiotic errors can be detected, thus making possible a presection of aneuploidy-free oocytes for fertilisation in an IVF programme[45]. The majority of PCR analyses for PGD have been performed on embryos for a single-gene disorder. Contamination of the PCR assay by extraneous DNA, however, may be a problem that can arise during the biopsy of at cleavage- or blastocyst-stage embryos, if sperm DNA is adhering to the zona pellucida. This contamination can be avoided by using ICSI as an ART procedure in PGD cycles.

Fluorescent in situ hybridisation (FISH) is a technique in which a fluorochrome-labelled DNA probe is hybridised with a specific segment of a chromosome and then visualised under fluorescence microscopy. While FISH is not useful for analysing disorders caused by single-gene defects, it is useful to access aneuploidy, to define mosaicism, and to determine gender.[46,47] Thus, the presence or absence, or the number of a particular chromosome in a biopsy specimen, be it a polar body or a blastomere, may be determined. In contrast to single-gene defects, numerical chromosome abnormalities occur de novo, and the only known risk factor is maternal age. Currently, probes for chromosomes X, Y, 13, 14, 15, 16, 18, 21, and 22 are being used simultaneously; this procedure has the potential of detecting 70% of aneuploidies. New probe colours are now being developed to avoid different chromosomes sharing one colour. Mixoploidy, however, appears to be a normal feature of embryos, and tetraploid cells usually end up in the trophectoderm and are not considered to be seriously detrimental to the embryo.[48]

New techniques under development

Comparative genome hybridisation can accurately determine total or partial aneusomy by loss or gain of DNA, by using a combination of PCR and FISH techniques. Test and control DNAs are detected by differently coloured fluorochromes, such as green and red. Using an image analysis system, the resulting intensity ratio of green to red fluorescence for each chromosome should reflect the number of homologous chromosomes present in the test DNA: 0 for nullisomies, 0.5 for monosomies, 1 for normal cells, 1.5 for trisomies, and so on.

Quantitative fluorescent PCR was developed originally for the molecular cytogenetic diagnosis of solid tumours by Kallioniemi et al.[49] The method can be used to detect chromosomal euploidies, provided that the sample contains 10 or more cells.

As an alternative to conventional FISH, spectral imaging involves the use of 24 painting probes, one for each chromosome. This technique can be applied to oocytes, first polar bodies, and blastomere metaphases. It is particularly suited for the painting of polar bodies for PGD of translocation,[50] because more than 90% of first polar bodies are at metaphase within 6 hours after egg retrieval. On the other hand, conventional karyotyping cannot be used on blastomeres, because an average of only 25% of blastomere nuclei show metaphase chromosomes after antimitotic treatment, and only few nuclei show banding-quality chromosomes.

In vitro maturation of human primary oocytes

The maturation of immature oocytes retrieved from an unstimulated ovary in the laboratory is a subject of intense study. Cha et al[51] of South Korea and Trounson et al[52] of Australia first described successful in vitro maturation of human oocytes that resulted in live births in 1991 and 1995, respectively. However, technical difficulties in collecting 5- to 12-mm sized antral-stage follicles from an unstimulated, non-polycystic ovary is a daunting task for the clinician. The harvest of fewer eggs (except in cases of polycystic ovary), practical differences in the culture requirements, retarded embryo development, and lower pregnancy rates than conventional IVF are problems that would require many years to overcome. If it ever becomes a clinical reality, in vivo oocyte maturation will greatly reduce the patient's drug costs, spare them daily injections of gonadotrophins, and reduce the incidence of ovarian hyperstimulation syndrome.

Cryopreservation of human ovarian tissue

The theoretical advantages of cryopreserving human ovarian tissue are obvious. A large number of primordial follicles can be collected in a piece of ovarian tissue and stored. Furthermore, primordial follicles appear less vulnerable to cryoinjury than growing or mature follicles because of their small size, low metabolic rate, cell stage (meiosis I of prophase), and absence of zona and cortical granules. A single ovarian graft has the potential to restore ovarian hormonal and ovulatory functions. Unlike eggs and embryos, however, ovarian tissue can serve as a reservoir for infection or cancer cells. To date, neither freeze-thaw

procedures nor transplantation procedures have been optimised for clinical applications.

Conclusion

While early success rates using IVF ranged from 10% to 15%,[53] improved technology, such as stimulation protocols and embryo culture systems, have more than doubled that rate.[54] Human IVF will continue to evolve from a scientific discipline for the treatment of human infertility to the application of molecular biology and genetics to treat or prevent an ever-growing range of human diseases and dysfunctions.

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