Human Reproduction Vol.21, No.2 pp. 518–523, 2006 doi:10.1093/humrep/dei350
Advance Access publication October 20, 2005.

A prospective study, using sibling oocytes, examining the effect of 30 seconds versus 90 minutes gamete co-incubation in IVF

Mona Bungum[1,2,3], Leif Bungum[1] and Peter Humaidan[1]

[1]The Fertility Clinic, Viborg Hospital (Skive), Resenvej 25, DK 7800 Skive, Denmark and [2]Fertility Centre, Scanian Andrology Centre, Malmö University Hospital, Malmö, Sweden

[3]Correspondence: Mail adr.: Fertility Clinic, Viborg Hospital (Skive), Resenvej 25, 7800 Skive, Denmark. E-mail: mona.bungum@sygehusviborg.dk

BACKGROUND: Traditionally oocytes have been exposed to sperm overnight, for 16–20 h. This long period of co-incubation, however, has been shown to create problems with high levels of reactive oxygen species (ROS), which may affect embryo viability and cause hardening of the zona pellucida. Recently, a positive effect of reducing the co-incubation time to 90–120 min was reported. The objective of this study was to evaluate whether a further reduction of the co-incubation period could benefit the outcome of IVF. METHODS: In this prospective study, 777 sibling oocytes from 81 women undergoing IVF were divided via alternate allocation to co-incubation for either 30 s (ultrashort co-incubation) (group A) or for 90 min (standard co-incubation) (group B). Endpoints were normal fertilization (two-pronuclear, 2PN), polyspermy (>2PN), embryo quality (EQ), clinical pregnancy (CP) and implantation (IR). RESULTS: The normal fertilization rates of the two groups were comparable: group A 58.6% versus group B 58.0%. Significantly lower rates of polyspermy were seen in group A compared to group B (2.8 versus 7.2%, *P* = 0.008). No statistically significant differences in EQ, CP or IR were seen. CONCLUSION: This is the first study demonstrating the achievement of good fertilization rates in IVF with ultrashort co-incubation. Significantly lower rates of polyspermy were seen in the group with ultrashort compared to the standard co-incubation group. Further studies are, however, needed in order to evaluate whether ultrashort co-incubation has any effect on the outcome of IVF.

Key words: fertilization/gamete co-incubation/IVF/polyspermy

Introduction

In human IVF, oocytes traditionally have been exposed to sperm overnight, for 16–20 h. This long period of co-incubation, however, has been shown to create problems with high levels of reactive oxygen species (ROS), which may affect the quality of embryos (Gianaroli *et al.*, 1996b; Dirnfeld *et al.*, 1999; Bedaiwy *et al.*, 2004) and cause hardening of the zona pellucida, known to negatively influence the implantation potential of the embryo (Waldenstrøm *et al.*, 1993; Gianaroli *et al.*, 1996a,b; Dirnfeld *et al.*, 2003). Moreover ROS is one of the main sources to DNA strand breaks in sperm (Aitken and Clarkson, 1987; Aitken *et al.*, 1989a,b). Although DNA-damaged sperm are able to fertilize oocytes (Ahmadi and Ng, 1999), high rates of DNA breaks are known to negatively influence fertility *in vivo* (Evenson *et al.*, 1999; Spanò *et al.*, 2000) as well as *in vitro* (Lopes *et al.*, 1998; Evenson and Jost, 2000; Larson *et al.*, 2000; Duran *et al.*, 2002; Morris *et al.*, 2002; Tomsu *et al.*, 2002; Benchaib *et al.*, 2003; Larson-Cook *et al.*, 2003; Saleh *et al.*, 2003; Bungum *et al.*, 2004; Gandini *et al.*, 2004; Virro *et al.*, 2004).

In several recent publications the effect of a reduction of the co-incubation time from 16–20 h to 90–120 min has been studied (Gianaroli *et al.*, 1996a,b; Coskun *et al.*, 1998; Quinn *et al.*, 1998; Dirnfeld *et al.*, 1999; Lin *et al.*, 2000; Lundqvist *et al.*, 2001; Kattera and Chen, 2003). Some of these have reported that better embryo quality can be obtained by shortening the co-incubation time, suggested to be a result of decreased levels of ROS present in the culture (Gianaroli *et al.*, 1996b; Dirnfeld *et al.*, 1999). Recently it was demonstrated in pigs that both penetration and blastocyst rates were improved by reducing the co-incubation time from 5 h to 10 min (Grupen and Nottle, 2000; Gil *et al.*, 2004).

Our study was initiated in order to investigate whether it was possible to further reduce the co-incubation period without reducing the fertilization rates and to study potential effects of the time reduction. The primary endpoints were normal fertilization (two- pronuclear, 2PN) and polyspermy rate (>2PN). Secondary endpoints were embryo quality (EQ), biochemical pregnancy (BP), clinical pregnancy (CP) and implantation rate (IR). Patients were used as their own controls by dividing sibling oocytes between the study group and the control group.

Materials and methods

Patients and study design

This prospective study was based on a cohort of consecutive infertile couples undergoing conventional IVF treatment at the Fertility Clinic, Viborg Hospital (Skive) during the period of December 2004 to May 2005. All couples in which the male partner had normal sperm concentration and motility according to the World Health Organization (WHO, 1999) were asked to participate in the study. All participants signed an informed consent. A total of 777 sibling oocytes from 81 women were allocated to co-incubation for either 30 s (ultrashort co-incubation, group A) or for 90 min (standard co-incubation, group B), so the patients were used as their own controls. While 389 oocytes were analysed in group A, 388 oocytes were anaysed in group B. Each patient contributed with one cycle.

IVF procedure

Procedures for hormonal treatment, oocyte retrieval, allocation, sperm and oocyte handling

Patients underwent pituitary down-regulation and hormonal treatment as previously described (Bungum *et al*., 2003). When at least one follicle had reached a diameter of ≥17 mm, 10 000 IU of HCG (Ovitrelle; Serono Nordic, Copenhagen, Denmark) was administered to induce final follicular maturation. Oocyte retrieval was performed 35 h after HCG injection by vaginal ultrasound-guided follicle aspiration. G-MOPS™ (Vitrolife, Gothenburg, Sweden) was used to rinse the oocytes. The oocytes were placed in G-Fert™ (Vitrolife) for incubation until exposure to sperm 2–3 h after oocyte retrieval. Semen samples were collected by masturbation at the day of oocyte retrieval. Semen analysis was performed according to the WHO (1999) guidelines. A standard density gradient centrifugation method, PureSperm, 45 and 90% (Nidacon Ltd, Gothenburg, Sweden) diluted in G-Sperm™ (Vitrolife), was used for sperm preparation. The washing procedure and the final dilution were performed in IVF-100™ (Vitrolife).

Immediately after OR the oocytes were alternately allocated to either ultrashort co-incubation (30 s) (group A) or to standard co-incubation (90 min) (group B) (Figure 1). A laboratory technician performed the random allocation, every second oocyte to each group. The allocation was not blinded. In both groups oocytes were incubated in G-Fert™ (Vitrolife) supplemented with sperm with a final concentration of 150 000/ml. In group A the co-incubation procedure was performed at the heating stage (37°C) in a laminar air flow hood and thereafter the oocytes were washed three times in G.1.3™ (Vitrolife), to remove sperm not attached to the cumulus–corona complex before further culture in G.1.3™ until time of assessment for fertilization. Group B oocytes were co-incubated in a humidified incubator at 37°C in a gas phase of 6% CO_2 and 5% O_2 and 89% N_2 for 90 min and thereafter washed three times in G.1.3™, before further culture in G.1.3™ until fertilization assessment.

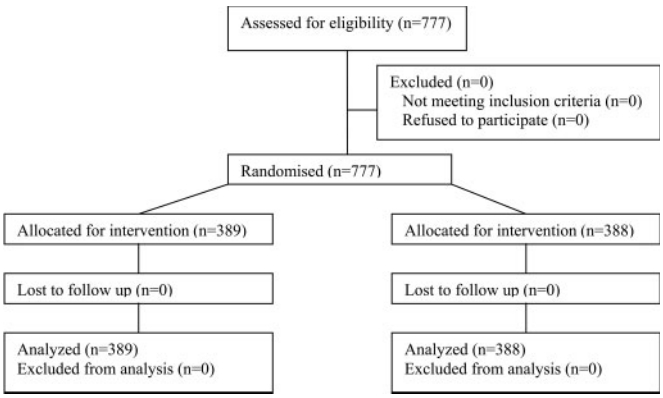

Figure 1. The flow of oocytes through the trial.

Assessment of fertilization, culture procedure, embryo morphology evaluation, cryopreservation and embryo transfer

Normal fertilization was characterized by two visible distinct pronuclei determined 18–20 h after insemination. Polyspermy was defined as more than two visible pronuclei. After assessment of fertilization zygotes were cultured in G1.3™ until embryo transfer day 2 or 3. A maximum of five fertilized oocytes were cultured in 50 µl media droplets under oil (Ovoil™). A gas phase of 6% CO_2 and 5% O_2 and 89% N_2 was used in a 37°C humidified incubator.

In addition to the number of blastomeres, the following morphological parameters were assessed on the morning of day 2 and prior to embryo transfer: (i) extracellular fragmentation: embryos with no fragmentation (grade 0); embryos with <10% fragmentation (grade 1); embryos with 10–20% fragmentation (grade 2); embryos with 20–50% fragmentation (grade 3); embryos with >50% fragmentation (grade 4); (ii) location of extracellular fragments: locally fragmented blastomeres (A1) versus dispersed fragmented blastomeres (A2); (iii) symmetry of blastomeres: equally sized symmetrical blastomeres (B1) versus unevenly sized blastomeres (B2); (iv) appearance of cytoplasm: homogeneous cytoplasm (C1) versus granulated or vacuolated cytoplasm (C2); (v) multinucleation: no multinucleate blastomeres present (D1) versus multinucleated blastomeres present (D2)

One or two embryos with the best morphology were selected for embryo transfer on day 2 or 3 after oocyte retrieval. This selection policy resulted in three embryo transfer groups: (i) one or two embryos from group A; (ii) one or two embryos from group B; (iii) mixed embryo transfer. All embryo transfers were performed in Embryo Glue™ (Vitrolife) with a Cook Soft 5000 catheter (Cook, Brisbane, Australia).

Strict criteria for cryopreservation were used. Only embryos with at least seven blastomeres and <20% intracellular fragments were cryopreserved on day 3.

Luteal phase support and pregnancy test

Patients received luteal phase support in the form of micronized progesterone vaginally, 90 mg once a day (Crinone 8%; Serono Nordic, Copenhagen, Denmark) starting on the day following oocyte retrieval and continuing until the day of the pregnancy test (i.e. day 12 after embryo transfer). A positive pregnancy test was defined by a plasma βHCG concentration >10 IU/l. A clinical pregnancy was defined as an intrauterine gestational sac with a heart beat 3 weeks after a positive HCG test. The implantation rate was calculated as the ratio of gestational sacs determined by ultrasound after 7 weeks in relation to the total number of embryos transferred.

Statistical methods

For statistical analysis Statistix, version 8 software (Tallhassee, USA) was applied using Pearson's χ^2-test. $P < 0.05$ was defined as being statistically significant.

Results

In all, 777 oocytes from 81 women were included in the study. In group A, 389 oocytes were analysed, and in group B, 388 oocytes were analysed. All relevant demographic data including female age, infertility diagnosis, oocytes retrieved, sperm concentration and progressive motility are given in Table I. The normal fertilization rates (2 PN) of the two groups were

Table I. Demographic data

Patients included (*n*)	81
Female age, mean ± SD (years)	32.8 ± 3.8
Infertility diagnosis, *n* (%)	
Tubal factor	41 (50.6)
Endometriosis	4 (4.9)
Unexplained	29 (35.8)
Anovulation	7 (8.6)
No. of oocytes retrieved per patient, mean ± SD	vb9.6 ± 5.0
Sperm concentration, mean ± SD (range) ($\times 10^6$/ml)	72.2 ± 43.2 (20.0–198.0)
Sperm progressive motility, mean ± SD (%)	68.8 ± 14.6

comparable; group A 58.6% versus group B 58% (not significant) (Table II). Significantly lower rates of polyspermy (>2PN) were seen in group A compared to group B (2.8 versus 7.2%, $P = 0.008$) (Table II). In seven cycles of group A and in seven cycles in group B no normal fertilization occurred. However, in four of the cycles there was normal fertilization in one of the groups, so only three cycles (3.7%) were cancelled due to total fertilization failure (data not shown). Six cycles (7.4%) were cancelled because of poor embryo development in both groups (data not shown). Regarding EQ, the number of embryos of grade 0 and 1 and the number of embryos cryopreserved in the two groups were comparable; 58.8 versus 67.6%, and 30.3 versus 28.0% respectively (not significant) (Table II).

Seventy-two of the 81 patients received embryo transfer (88.9%). When dividing the patients into three groups, those who received: (i) one or two embryos from group A; (ii) one or two embryos from group B; (iii) mixed transfer; no statistical significant differences were seen regarding BP, CP or IR (Table III).

Table II. Oocytes, fertilization and embryo quality

	Group A (30 s)	Group B (90 min)	*P*[a]
Oocytes for insemination, *n*	389	388	NS
Oocytes normally fertilized (2PN), *n* (%)	228/389 (58.6)	225/388 (58)	NS
Oocytes with polyspermy (>2PN), *n* (%)	11/389 (2.8)	28/388 (7.2)	0.008
Embryos grade 0 or 1, *n* (%)	134/228 (58.8)	152/225 (67.6)	NS
Embryos cryopreserved, *n* (%)	69/228 (30.3)	63/225 (28.0)	NS

[a]Pearson's χ^2.
PN = pronuclei; NS = not significant.

Discussion

The primary goal of our study was to investigate whether an oocyte–sperm co-incubation time of 30 s is long enough to achieve good fertilization rates in human IVF. To the best of our knowledge this is the first study demonstrating successful fertilization with an ultrashort co-incubation period. Moreover, we have shown that the rate of polyspermy can be effectively decreased.

Data from several previous publications have contributed to the set-up of this study. Firstly, the study was inspired by recent data from IVF in pigs where improved penetration and blastocyst rates after a co-incubation time as short as 10 min were seen (Grupen and Nottle, 2000; Gil *et al.*, 2004). Secondly, data demonstrating that the human sperm bind to the cumulus–oocyte complex (COC) within 11 min after coitus or *in vitro* insemination also have contributed to the set-up of our study (Wasserman, 1987, 1988; Gianaroli *et al.*, 1996a). Lastly, we have been motivated by findings from our own study (Bungum *et al.*, 2004) where IVF cycles (90 min co-incubation) in couples with high rates of sperm DNA breaks despite normal conventional sperm parameters (WHO, 1999) resulted in significantly lower pregnancy rates than ICSI cycles. Although levels of ROS were not measured, one could suspect high levels of ROS present in the IVF culture to be a possible explanation for the difference in favour of ICSI, as previous studies have shown a negative correlation between ROS and fertilization rates (Krausz *et al.*, 1994; Agarwal *et al.*,2005), embryo quality (Nasr-Esfahani *et al.*, 1990) and outcome of assisted reproduction treatment (Saleh *et al.*, 2003). Although the issue of sperm chromatin integrity has been extensively explored, the complete aetiology of sperm DNA damage in infertile couples is unknown. Among several other questions it is unclear whether sperm DNA damage is a pre-testicular or a post-testicular event (Koopman *et al.*, 1994; Glander and Schaller, 1999; Barroso *et al.*, 2000; Oosterhuis *et al.*, 2000; Duru *et al.*, 2001a,b; Schuffner *et al.*, 2001, 2002; Shen *et al.*, 2002; Moustafa *et al.*, 2004). In a small study of seven fertile donors, the authors conclude that ejaculated sperm are incapable of initiating apoptosis (Lachaud *et al.*, 2004). However, the mechanisms causing sperm DNA damage, in fertile and infertile individuals may differ. Although storing and processing are two different procedures, Zini *et al.* (2000) demonstrated that infertile men have a 5-fold higher likelihood of having increased DNA denaturation after semen processing than fertile men. Since no previous reports on ultrashort co-incubation exist, the present study must be seen as a pilot study and consequently no power analysis was performed.

Table III. Pregnancy and implantation rates

	Group A (30s) (transfer of one or two embryos)	Group B (90 min) (transfer of one or two embryos)	Mixed embryo transfer (transfer of one embryo from each group)	*P*
Embryo transfer (*n*)	25	21	26	NS
Positive HCG, *n* (% per embryo transfer)	8 (32)	11 (52.4)	12 (46.2)	NS
Clinical pregnancies, *n* (% per embryo transfer)	7 (28)	8(38.1)	9 (34.6)	NS
Implantation rate, *n* (%)	10 /40 (25)	13/35 (37.1)	13/52 (25)	NS

NS = not significant.

Although we arbitrarily selected a co-incubation time as low as 30 s, we estimated the risk of total fertilization failure for a patient to be low, as half of the oocytes were co-incubated according to our standard procedure. The results showed that the normal fertilization rates (2PN) of the two groups were comparable: group A, 58.6% versus group B 58.0%; and that only 3.7% of the patients experienced total fertilization failure. We only included couples where the male partner had normal sperm concentration and motility according to the WHO (1999), and therefore, before recommending ultrashort co-incubation to patients with male factor, further studies should be performed. However, it is important to keep in mind that although the traditional sperm parameters are extensively used in the diagnostics and treatment of infertility, the parameters are poorly standardized (Giwercman *et al.*, 1999), subjective (Auger *et al.*, 2000), and not powerful predictors of fertility (Bonde *et al*., 1998; Zinaman *et al*., 2000; Guzick *et al*., 2001).

Regarding polyspermy, a significantly lower rate (>2 PN) was seen in group A compared to group B (2.8 versus 7.2%). In previous studies polyspermy has been related to the maturation status of the oocytes (Van Der Ven *et al.*, 1985; Angell *et al.*, 1986; Plachot *et al.*, 1998), and in the present study there was a difference of 90 min in sperm–oocyte exposure, so theoretically there could be more mature oocytes in the control group. Polyspermy has also been linked to the use of a high concentration of capacitated sperm at the site of fertilization and suboptimal *in vitro* conditions (Hunter, 1990; Niwa and Wang, 2001; Wang *et al.*, 2003). In IVF, oocytes are exposed to very high number of sperm, which can cause simultaneous sperm penetrations, whereas in normal conception the oviduct isthmus serves as a sperm reservoir and regulates the number of sperm reaching the site of fertilization, which ensures normal fertilization. In the present study, it is likely that the sperm selection mechanisms have been stricter in the study group than in the control group.

Our secondary goal with the study was to investigate whether ultrashort co-incubation time could benefit embryo quality, pregnancy and implantation, but no statistically significant differences regarding embryo quality, implantation and pregnancy were seen. Unfortunately, by choosing the one or two embryos with best morphology for embryo transfer, we obtained three subgroups with relatively few patients, which makes it difficult to evaluate pregnancy and implantation outcome. Surprisingly and opposite to our hypothesis, however, we observed a tendency to a lower pregnancy and implantation rate in the study group compared to the standard co-incubation group. According to our hypothesis the oocytes in the study group should suffer less oxidative damage than those in the control group. However, as ROS also is generated by somatic cells, a possible explanation for the result could be that the granulosa cells in the COC contributed to ROS production. Sperm carry freely diffusible hyaluronidase that aims to loosen the bindings between the granulosa cells which enable the spermatozoon to pass through the COC (Swyer, 1947; Schrader and Leuchenberger, 1951). In the present study we observed that while a minimal amount of the COC were left on the oocytes after 90 min exposure to sperm, oocytes incubated for 30 s had a fully intact COC, which also may contribute to production of ROS. Moreover, a recent study by Kattera and Chen (2003) demonstrated that cumulus and corona cells release estradiol, which in turn may have a direct toxic effect on the embryo (Valbuena *et al.*, 2001). Consequently, one might speculate whether a mechanical or enzymatic denudation of the oocytes prior to ultrashort co-incubation could minimize the problems. However, a disadvantage of removal of COC could be a disturbance of the important communication between the oocyte and the COC (Canipari, 2000).

From a practical point of view, an advantage with the ultrashort co-incubation is that the procedure can be handled more effectively in one step, instead of two as in the standard procedure. Fewer incubator openings could minimize embryonic stress during the culture period.

In conclusion, this is the first study to demonstrate that a co-incubation time as short as 30 s is long enough to obtain good fertilization rates in human IVF. In addition we have shown that the rate of polyspermy can be effectively minimized. Further studies are, however, needed before firm conclusions regarding the relationship between gamete co-incubation times, embryo quality and pregnancy outcome can be reached.

Acknowledgement

The contribution of the laboratory staff in the Fertility Clinic, Viborg Hospital (Skive), in particular Dorthe Tudborg, is gratefully acknowledged.

References

Agarwal A, Allamaneni S, Nallella K, George A and Mascha E (2005) Correlation of reactive oxygen species levels with the fertilisation rate after in vitro fertilisation: a qualified meta-analysis. Fertil Steril 84,228–231.

Ahmadi and Ng (1999) Fertilizing ability of DNA-damaged spermatozoa. J Exp Zool 284,696–704.

Aitken RJ and Clarkson JS (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 81,459–469.

Aitken RJ, Clarkson JS and Fishel S (1989a) Generation of reactive oxygen species, lipid peroxidation and human sperm function. Biol Reprod 40,183–197.

Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS and Wu FCW (1989b) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. J Androl 10,214–220.

Angell RR, Templeton AA and Aitken RJ (1986) Chromosomal studies in human in vitro fertilisation. Hum Genet 72,333–339.

Auger J, Eustache F, Ducot B, Blandin T, Dandin M, Diaz I, Matribi SE, Gony B, Keskes L and Kolbezen M et al (2000) Intra- and inter-individual variability in human sperm concentration, motility and vitality during a workshop involving ten laboratories. Hum Reprod 15,2360–2368.

Barroso G, Morshedi M and Oehninger S (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod 15,1338–1344.

Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J and Agarwal A (2004) Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 82,593–600.

Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H and Guerin JF (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod 18,1023–1028.

Bonde JP, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Henriksen TB, Scheike T, Giwercman A, Olsen J and Skakkebaek NE (1998) Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. Lancet 352,1172–1177.

Bungum M, Bungum L, Humaidan P and Yding Andersen C (2003) Day 3 versus day 5 embryo transfer; a prospective randomised study. Reprod Biomed Online 7,98–104.

Bungum M, Humaidan P, Spano M, Jepson K, Bungum L and Giwercman A (2004) The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. Hum Reprod 19,1401–1408.

Canipari R (2000) Oocyte–granulosa cell interactions. Hum Reprod Update 6,279–289.

Coskun S, Roca GL, Elnour AM, al Mayman H, Hollanders JM and Jaroudi KA (1998) Effects of reducing insemination time in human in vitro fertilisation and embryo development by using sibling oocytes. J Assist Reprod Genet 15,605–608.

Dirnfeld M, Bider D, Koifman M, Calderon I and Abramovici H (1999) Shortened exposure of oocytes to spermatozoa improves in vitro fertilisation outcome: a prospective, rendomised, controlled study. Hum Reprod 14,2562–2564.

Dirnfeld M, Shiloh H, Bider D, Harari E, Koifman M, Lahav-Baratz S and Abramovici H (2003) A prospective randomised controlled study of the effect of short coincubation of gametes during insemination on zona pellucida thickness. Gynecol Endocrinol 17,397–403.

Duran EH, Morshedi M, Taylor S and Oehninger S (2002) Sperm DNA quality predicts intrauterine insemination outcome—a prospective cohort study. Hum Reprod 17,3122–3128.

Duru NK, Morshedi M and Oehninger (2001a) Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil Steril 75,263–268.

Duru NK, Morshedi M, Schuffner A and Oehninger S (2001b) Cryopreservation–thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. J Androl 22,646–651.

Evenson DP and Jost LK (2000) Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci 22,169–189.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P and Claussen OP (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 14,1039–1049.

Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, Leter G, Ciriminna R, Culasso F, Dondero F and Lenzi A et al (2004) Full time pregnancies achieved with ICSI despite high levels of sperm chromatin damage. Hum Reprod 19,1409–1417.

Gianaroli L, Cristina Magli M, Ferraretti AP, Fiorentino A, Tosti E, Panzella S and Dale B (1996a) Reducing the time of sperm–oocyte interaction in human in-vitro fertilisation improves the implantation rate. Hum Reprod 11,166–171.

Gianaroli L, Fiorentino A, Magli MC, Ferraretti AP and Montanaro N (1996b) Prolonged sperm–oocyte exposure and high sperm concentration affect human embryo viability and pregnancy rate. Hum Reprod 11,2507–2511.

Gil MA, Ruiz M, Vazquez JM, Roca J, Day BN and Martinez EA (2004) Effect of short periods of sperm–oocyte coincubation during in vitro fertilisation on embryo development in pigs. Theriogenology 62,544–552.

Giwercman A, Spanò M, Lahdetie J and Bonde JP (1999) Quality assurance of semen analysis in multicenter studies. Asclepios Scand J Work Environ Hlth 25(Suppl 1),3–25.

Glander HJ and Schaller J (1999) Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Mol Hum Reprod 5,109–115.

Grupen CG and Nottle MB (2000) A simple modification of the in vitro fertilisation procedure. Theriogenology 53,422.

Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf PP and Hill JA et al (2001) Sperm morphology, motility and concentration in infertile and fertile men. New Engl J Med 345,1388–1393.

Hunter RHF (1990) Fertilisation of pig eggs in vitro and in vivo. J Reprod Fertil Suppl 40,211–226.

Kattera S and Chen C (2003) Short coincubation of gametes in in vitro fertilisation improves implantation and pregnancy rates: a prospective, randomised, controlled study. Fertil Steril 80,1017–1021.

Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST and van Oers MH (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84,1415–1420.

Krausz C, Mills C, Rogers S, Tan SL and Aitken RJ (1994) Stimulation of oxidant generation by human sperm suspensions using phorbol esters and phormyl peptides: relationships with motility and fertilisation in vitro. Fertil Steril 62,599–605.

Lachaud C, Tesarik J, Canadas ML and Mendoza C (2004) Apoptosis and necrosis in human ejaculated spermatozoa. Hum Reprod 19, 607–610.

Larson KL, DeJonge CJ, Barnes AM, Jost LK and Evenson DP (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. Hum Reprod 15,1717–1722.

Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET and Evenson DP (2003) Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 80,895–902.

Lin SP, Lee RK, Su JT, Lin MH and Hwu YM (2000) The effect of brief gamete coincubation in human in vitro fertilisation. J Assist Reprod Genet 17,344–348.

Lopes S, Sun JG, Jurisicova A, Meriano J and Casper RF (1998) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilisation in intracytoplasmic sperm injection. Fertil Steril 69,528–532.

Lundqvist M, Johansson U, Lundquist Ø, Milton K, Westin C and Simberg N (2001) Reducing the time of coincubation of gametes in human in vitro fertilisation has no beneficial effects. Reprod Biomed Online 3,21–24.

Morris ID, Ilott S, Dixon L and Brison DR (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilisation and embryo development. Hum Reprod 17,990–998.

Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ and Agarwal A (2004) Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod 19,129–138.

Nasr-Esfahani MH, Aitken JR and Johnson MH (1990) Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. Development 109,501–507.

Niwa WH and Wang WH (2001) Cortical granules and cortical reaction in pig oocytes. In Miyamoto H and Manabe N (eds) Reproductive Biotechnology. Update and its Related Physiology Tokyo: Elsevier Scientific Publishers, 2001, 27–34.

Oosterhuis GJE, Mulder AB, Kalsbeek-Batenburg E, Lambalk CB, Schoemaker J and Vermes I (2000) Measuring apoptosis in human spermatozoa: a biological assay for semen quality? Fertil Steril 74,245–250.

Plachot M, Veiga A, Montagut J, de Grouchy J, Calderon G, Lepretre S, Junca AM, Santalo J, Carles E and Mandelbaum J et al (1998) Are clinical and biological IVF parameters correlated with chromosomal disorders in early life: a multi-centric study. Hum Reprod 3,627–635.

Quinn P, Michael LL, Minh Ho, Bastuba M, Hendee F and Brody SA (1998) Confirmation of the beneficial effects of brief coincubation of gametes in human in vitro fertilisation. Fertil Steril 69,399–402.

Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR and Thomas AJ (2003) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility Fertil Steril 79(Suppl 3),1597–1605.

Schrader F and Leuchenberger C (1951) The cytology and chemical nature of some constituents of the developing sperm. Chromosoma 4,404–428.

Schuffner A, Morshedi M and Oehninger S (2001) Cryopreservation of fractionated, highly motile human spermatozoa: effect on membrane phosphatidylserine externalization and lipid peroxidation. Hum Reprod 16,2148–2153.

Schuffner A, Morshedi M, Vaamonde D, Duran EH and Oehninger S (2002) Effect of different incubation conditions on phosphatidylserine. J Androl 23,194–201.

Shen HM, Dai J, Chia SE, Lim A and Ong CN (2002) Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Hum Reprod 17,1266–1273.

Spanò M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E and Leter G (2000) Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril 73,43–50.

Swyer GIM (1947) The hyaluronidase content of semen. Biochem J 41,409–413.

Tomsu M, Sharma V and Miller D (2002) Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. Hum Reprod 17,1856–1862.

Valbuena D, Martin J, De Pablo JL, Remohi J and Pellicer A (2001) Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. Fertil Steril 76,962–968.

Van Der Ven HH, Al-Hasani S, Dietrich K, Hammerich U, Lehman F and Krebbs D (1985) Polyspermy in in vitro fertilisation of human oocytes. Pregnancy and possible causes. Ann NY Acad Sci 442,88–95.

Virro MR, Larson-Cook KL and Evenson DP (2004) Sperm chromatin structure assay (SCSA) parameters are related to fertilisation, blastocyst development,

and ongoing pregnancy in in vitro fertilisation and intracytoplasmic sperm injection cycles. Fertil Steril 81,1289–1295.

Waldenstrøm U, Hamberger L, Nilsson L and Ryding M (1993) Short-time sperm–egg exposure to prevent zona hardening. J Assist Reprod Genet 10,190.

Wang WH, Day BN and Wu GM (2003) How does polyspermy happen in mammalian oocytes? Micro Res Tech 61,335–341.

Wasserman PM (1987) The biology of chemistry of fertilisation. Science 285,553–560.

Wasserman PM (1988) Fertilisation in mammals. Sci Am 259,78–84.

World Health Organization (1999) WHO Laboratory Manual for the Examination of Human Sperm and Sperm–Cervical Mucus Interaction. Cambridge University Press, Cambridge, UK.

Zinaman MJ, Brown CC, Selevan SG and Clegg ED (2000) Semen quality and human fertility: a prospective study with healthy couples J Androl 21,145–153.

Zini A, Finelli A, Phang D and Jarvi K (2000) Influence of semen processing technique on human sperm DNA integrity. Urology 56,1081–1084.

Submitted on July 31, 2005; resubmitted on September 5, 2005; accepted on September 12, 2005