Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI

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BACKGROUND: Failed fertilization occurs in 2–3% of ICSI cycles and is mainly due to lack of oocyte activation. Heterologous ICSI of patient's sperm in mouse oocytes allows discrimination between sperm- and oocyte-related aetiologies of activation failure. Assisted oocyte activation (AOA) by Ca-ionophore treatment can initiate fertilization in subsequent therapeutic ICSI. We report on diagnosis and clinical treatment in 17 patients with previously failed fertilization. METHODS: Sperm from patients were injected into mature mouse oocytes. Activation capacity was assessed by 2-cell formation (mouse oocyte activation test, MOAT). When no activation occurred, it was assumed that the spermatozoon was deficient; otherwise an oocyte-related factor was suspected. In a subsequent ICSI cycle, AOA was done by ICSI with CaCl$_2$ followed by a Ca^{2+} ionophore exposure. Fertilization was checked 16–20 h later. Embryo transfer was on day 2 or 3. RESULTS: MOAT showed sperm-related activation deficiency in six globozoospermic patients and two patients with extreme oligoasthenoteratozoospermia. One patient with small sperm acrosomes had a normal activation percentage. In eight other patients, the MOAT revealed a relatively normal activation capacity of the sperm, indicating an oocyte-related defect. After AOA, fertilization rates were 77 and 71% in the sperm- and oocyte-related groups respectively. Five pregnancies were achieved in the globozoospermia group and three in cases of oocyte-related activation failure. CONCLUSIONS: Assisted oocyte activation enables normal fertilization and pregnancy in sperm- and oocyte-related fertilization failure.

Key words: calcium ionophore/fertilization failure/heterologous ICSI/oocyte activation

Introduction

ICSI was introduced to overcome severe male infertility. The ICSI procedure results in an average fertilization rate of 70% (Ben-Yosef and Shalgi, 2001) but in rare cases fertilization fails due to lack of oocyte activation (Sousa and Tesarik, 1994; Kovacic and Vlaisavljevic, 2000). Failed fertilization occurs in 2–3% of ICSI cycles (Mahutte and Arici, 2003). Artificial induction of oocyte activation can be of benefit in those cases. Successful assisted oocyte activation (AOA) by calcium ionophore treatment has been reported in five case reports (Rybouchkin *et al.*, 1997; Kim *et al.*, 2001; Eldar-Geva *et al.*, 2003; Chi *et al.*, 2004; Murase *et al.*, 2004). Electrical oocyte activation (Yanagida *et al.*, 1999) and a modified ICSI technique to overcome activation failure (Tesarik *et al.*, 2002; Ebner *et al.*, 2004) have also been successfully applied.

Oocyte activation is characterized by a two-step pattern of rises in intracellular Ca^{2+} concentrations. A first Ca^{2+} rise (trigger) originates from the oocyte cortex after sperm–oocyte membrane interaction and is followed 30 min later by a series of shorter Ca^{2+} transients of high amplitude that continue for 3–4 h (oscillator) (Tesarik *et al.*, 2000). The oscillator function is dependent on the release of a sperm-associated oocyte activation factor that conditions the oocyte to sustain repetitive Ca^{2+} releases from intracellular stores (Ben-Yosef and Shalgi, 2001). This sperm factor is sensitive to heat, is not species-specific and can control the frequency of oscillations. The repetitive nature of this signal is essential for complete oocyte activation. Tesarik *et al.* (2002) have also shown that not only a sperm factor but also an oocyte factor is involved in the activation oscillator mechanism. To distinguish between deficiency of the oocyte-activating capacity of sperm or the inability of oocytes to respond to penetrated sperm, a heterologous ICSI model can be used as a diagnostic tool (Rybouchkin *et al.*, 1996).

We applied heterologous ICSI of human sperm into mouse oocytes (mouse oocyte activation test, MOAT) for diagnosing sperm- or oocyte-related activation deficiencies in a group of 17 patients. AOA in a subsequent ICSI cycle was done by Ca^{2+}-ionophore treatment. We were able to normalize fertilization rates after ICSI and to obtain pregnancies in cases of sperm- or oocyte-related activation deficiencies.

Materials and methods

Patient population

Indications for a diagnostic heterologous ICSI included globozoospermia ($n = 6$), a small or absent acrosome ($n = 1$), total fertilization failure ($n = 4$), a low fertilization rate (mean 11%) ($n = 4$) and a low fertilization rate followed by poor embryo development ($n = 2$). We obtained Institutional Review Board approval for treatment of these patients with Ca-ionophore.

Heterologous ICSI or mouse oocyte activation test

B6D2 F1 hybrid female mice aged 7–14 weeks were induced to superovulate with 8–10 IU eCG (Folligon; Intervet, The Netherlands) followed by 8–10 IU of hCG (Chorulon, Intervet) 48 h later. Collection of metaphase II oocytes was done 13–14 h post-hCG. Potassium-simplex optimized medium (KSOM) (Lawitts and Biggers, 1991) containing 0.2 mmol/l glucose and supplemented with 0.4% bovine serum albumin (BSA; MP Biomedicals, Belgium) was used as oocyte and embryo culture medium. Cumulus–oocyte complexes were treated with 200 IU hyaluronidase (type VIII)/ml for 5 min to disperse the surrounding cumulus cells. A sharp needle was used to make a tangential slit in the zona pellucida at 12 o'clock (partial zona dissection, PZD). The chromosome–spindle complex, visible as a translucent region in the ooplasm, was located at 3–4 o'clock to avoid the spindle complex being destroyed during injection later on.

Fresh or frozen–thawed sperm from patients were washed twice by centrifugation at 1600 rpm for 10 min. Only motile sperm were selected for injection. For each MOAT, four experimental groups were set up: (i) injection with test-case sperm (30 oocytes); (ii) injection with donated sperm from a man with proven fertilization (positive control) (30 oocytes); (iii) sham injection of medium (negative control) (20 oocytes); (iv) non-manipulated oocytes to examine the occurrence of spontaneous parthenogenetic activation (medium control) (20 oocytes). Sperm were resuspended in injection medium consisting of KSOM–HEPES and an equal volume of 8% polyvinylpyrrolidone (PVP; ICSI-100; Vitrolife Scandinavia) in a 5 µl central drop. Oocytes were put in microdrops consisting of KSOM–HEPES supplemented with 20% fetal bovine serum (Gibco BRL; Invitrogen, Belgium). Injection was performed at 15–17°C as described elsewhere (Rybouchkin *et al.*, 1996). Motile sperm were immobilized by touching their tails with a blunt injection needle against the bottom of the dish. After deep invagination of the oocyte membrane with the injection needle (inner diameter 5–6 µm) through the slit made by PZD, the oolemma was broken by gentle suction. Cytoplasm was aspirated and then returned into the oocyte together with the spermatozoon. In the negative control group, sham injection of injection medium (KSOM-HEPES + PVP) was performed. Oocyte activation was assessed around 44 h post hCG by examining the percentage of 2-cell formation (number of 2-cells versus the number of surviving injected oocytes). As a standard, the positive control must show >90% 2-cell formation while both the negative control and medium control groups must show <10% 2-cell formation before MOAT results can be considered as reliable.

Assisted oocyte activation in clinical ICSI

The ionophore (ionomycin; cat. no. 159611, MP Biomedicals) was dissolved in cell culture-tested dimethylsulphoxide (DMSO; Sigma–Aldrich Chemie, Belgium) at a concentration of 1 mmol/l stock solution. The final solution containing 10 µmol/l ionophore was prepared just before ICSI by diluting the stock solution with Cook Cleavage medium (Cook Ireland Ltd, Ireland). For AOA, an immobilized sperm was first aspirated in the injection needle and brought to the tip of the needle. In a separate drop, 5 pl of 0.1 mol/l $CaCl_2$ was aspirated and injected along with the spermatozoon in the ooplasm. Thirty minutes after injection, a first Ca^{2+}-ionophore treatment was done during 10 min. Oocytes were then washed with Cook Cleavage medium and put in the incubator at 37°C in a 6% CO_2 air atmosphere. After another 30 min, the Ca^{2+} ionophore treatment was repeated for 10 min. Oocytes were washed and placed in Cook Cleavage medium in the incubator. Oocytes were checked for pronuclei 16–20 h after ICSI. Embryo transfer was carried out on day 2 or 3. Pregnancy was detected by measuring serum hCG on two independent occasions ≥15 days after embryo replacement. Clinical pregnancy was determined by observation of a gestational sac with fetal heartbeat on transvaginal ultrasound at 6–7 weeks of pregnancy.

Results

Table I gives an overview of the percentage of activation after MOAT, the advised clinical strategy, fertilization rates and pregnancies after AOA in the different patient groups with a history of failed fertilization.

Globozoospermia

Six couples had primary infertility caused by complete globozoospermia, which is characterized by round-headed sperm without an acrosome (type I globozoospermia) and moderate to severe asthenozoospermia (4–40% motile sperm). MOAT results showed complete failure of oocyte activation in five globozoospermic patients and almost no activation in the sixth (10% of oocytes activated) while the average activation rate in the positive controls was 95%. AOA was done on all oocytes during the following ICSI attempts in these six patients. In 12 cycles, 167 mature

Table I. Results of heterologous ICSI (MOAT%), the advised strategy and the outcome after application of assisted oocyte activation (AOA) during ICSI in terms of percentage fertilization and achieved pregnancies in the different patient groups

Patients	No.	History (%)	MOAT(%)	Strategy	Outcome AOA (%)	No. pregnant
Globozoospermia	6	–	0–10	All	77	5
Abnormal acrosomes	1	–	95	No AOA	–	1
Total fertilization failure in own centre	3	0	0–9 (2) or >90	All	76	0
Total fertilization failure in other centre	1	0	94	Half	67	0
Low fertilization	4	11	65–97	All	79	2
Low fertilization + embryo arrest	2	25	72/88	Half	60/67	1

History(%): fertilization % before MOAT and AOA.
Strategy: 'All': AOA on all oocytes during ICSI; 'Half': AOA on half of the number of oocytes during ICSI.
MOAT(%): number of 2-cells as a proportion of the number of surviving injected oocytes after MOAT = activation percentage.

oocytes were injected of which 128 oocytes were normally fertilized (77%). In one couple, two consecutive pregnancies were established from fresh embryo transfers. Another couple achieved two consecutive pregnancies from two fresh ICSI cycles (one singleton and a twin); in the third and fourth couple two singleton pregnancies were established from a fresh transfer; and in the fifth couple a singleton pregnancy was achieved from a frozen embryo transfer.

Small or absent acrosomes

MOAT was performed in a case of male infertility where the sperm analysis revealed sperm with very small or absent acrosomes. Because the MOAT resulted in normal oocyte activation (95%), no AOA was proposed. In fact, normal fertilization was obtained by a routine ICSI procedure and two consecutive pregnancies were achieved after transfer of two good quality embryos.

Total fertilization failure

In two couples with a severe male factor no fertilization was obtained by routine ICSI. The MOAT showed defective oocyte activation capacity of the sperm (0–9%). AOA was done during five subsequent ICSI attempts in these two couples. Twenty-five of 32 mature oocytes showed normal fertilization (78%) but no pregnancy was established.

In another couple which had no oocytes fertilized in two ICSI attempts, the MOAT revealed a normal oocyte activation capacity of the patients' sperm (94% activation). With AOA in two consecutive cycles a normal fertilization rate was obtained (14 out of 19 oocytes, 74%) but pregnancy was not established.

In a fourth couple with failed fertilization after ICSI (none of five mature oocytes) in another centre, the MOAT showed a normal activation capacity of the sperm (94% activation). During the following ICSI attempt, 18 mature oocytes were harvested, seven of nine were normally fertilized without AOA, while six of nine oocytes were fertilized using AOA. One good quality embryo originating from ICSI without AOA was transferred but no pregnancy was established.

Near fertilization failure

Four couples presented at our Infertility Centre because of poor fertilization in previous ICSI attempts elsewhere. From a total of 118 mature oocytes, retrieved in 12 cycles, only 11 (9%) were reportedly normally fertilized. The MOAT with the patients' sperm gave mixed results varying from a normal (97%) to a reduced oocyte activation capacity (65, 87 and 74%). In subsequent ICSI cycles, AOA was applied. The overall fertilization rate was 79% (77 out of 97 mature oocytes) and two couples achieved a singleton pregnancy.

Low fertilization rate and embryo arrest

A first patient was referred because of poor fertilization results in two previous ICSI cycles.

In the first attempt, only two oocytes were retrieved and none of them fertilized. In the second attempt, two out of six oocytes showed normal fertilization but were arrested at the pronuclear stage. The MOAT indicated an 88% activation

capacity of the patient's sperm compared to 100% in the positive control. We therefore advised to try AOA in a subsequent cycle. Three out of five oocytes showed normal fertilization but they became temporarily arrested at the pronuclear stage and a delayed and poor embryo development was observed 2 days later. No pregnancy was established in this couple after transfer of two embryos.

The second couple also had a history of poor fertilization and embryonic developmental arrest in three previous ICSI attempts before presenting at our clinic. Because the MOAT showed a decreased activation capacity (72%) of the patient's sperm compared to the positive control (91%), after MOAT, the couple was advised to undergo AOA on all oocytes if the number of oocytes was <10 and AOA on half of the oocytes if the number was >10. In the first cycle, only three mature oocytes were obtained of which two oocytes were normally fertilized after AOA. No pregnancy was achieved from the transfer of one excellent quality embryo. In the second attempt, 13 oocytes were retrieved. ICSI combined with AOA was performed on nine oocytes and four oocytes were treated with ICSI alone. After AOA, six out of nine oocytes were normally fertilized and four embryos of excellent quality were obtained of which two were selected for transfer. Four embryos were frozen. No pregnancy was achieved. Of four oocytes fertilized without assisted activation, two embryos of excellent quality were obtained and frozen for further treatment. Embryo transfer of two thawed (origin with or without AOA not known) embryos resulted in a twin pregnancy.

Discussion

Our results indicate that AOA by Ca^{2+} ionophore is efficacious for managing the rare cases of fertilization failure after ICSI. We further demonstrated that the MOAT can differentiate between sperm- and oocyte-related factors in fertilization failure and that this test has some prognostic value. The best results can be expected when the MOAT test indicates a deficiency of the sperm oocyte-activating factor as in cases of globozoospermia. When the MOAT is normal it is assumed that the fertilization failure is due to an undisclosed oocyte factor. Poor or absent fertilization in these cases could also be the result of some transient biological reasons or technical imperfections. Indeed, in several patients having had failed ICSI we were able to obtain normal fertilization without AOA.

A two-step mechanism, called the trigger and oscillation function, is involved in triggering and maintaining oocyte activation (Tesarik et al., 2002). The initial Ca rise, which is released from internal stores, is the trigger and is initiated by a receptor-mediated interaction between the spermatozoon and the oocyte plasma membrane. During ICSI this natural trigger is replaced by a so-called 'pseudotrigger' whereby a massive influx of Ca^{2+} into the oocyte is provoked by the injection procedure itself. The second function, the oscillator, is characterized by the development of Ca^{2+} oscillations, resulting from the release of a soluble sperm factor into the oocyte cytoplasm. The oscillator drives the oocyte's internal

calcium stores rendering them capable of supporting the ongoing, largely autonomous series of Ca oscillations for several hours (Tesarik and Mendoza, 1999; Ben-Yosef and Shalgi, 2001). In ICSI, the first Ca^{2+} rise starts 20–30 min after ICSI and originates from the oocyte cortex rather than from the vicinity of the injected sperm head. This Ca^{2+} rise alone is insufficient to fully activate the oocyte (Tesarik and Sousa, 1995). To sustain the oscillation function, sperm demembranization is necessary to facilitate the liberation of the cytosolic sperm factor responsible for the oscillator function (Dozortsev *et al.*, 1997; Yanagida *et al.*, 2001).

In the literature there are reports that success rates of ICSI in cases of globozoospermia are rather variable ranging from total fertilization failure to almost normal fertilization rates with the establishment of pregnancies without any AOA during ICSI (Stone *et al.*, 2000). Overall, fertilization rates remain poor using round-headed sperm for ICSI (Nakamura *et al.*, 2002) and assistance during ICSI is wanted. In patients with globozoospermia, heterologous ICSI provided evidence for a lack of the sperm factor. Battaglia *et al.* (1997) reported that apart from low fertilization rates associated with the use of round-headed sperm, cleavage rates were also compromised and they suggested that these sperm may lack normal centrosomes. After application of AOA during clinical ICSI, we were able to restore fertilization and embryo cleavage and developmental rates were brought to a normal level in these globozoospermic patients.

It has been demonstrated that Ca^{2+} oscillations in fertilized oocytes regulate not only short-term but also long-term developmental events (Ducibella *et al.*, 2002). It is thus possible that the developmental potential of these embryos may be compromised by not having a $[Ca^{2+}]_i$ signalling pattern that is optimal. This raises the question whether patients with embryos that show developmental arrest may also not be helped by AOA.

The first study in which an oocyte-borne oocyte activation deficiency was unequivocally recognized and efficiently treated was reported recently (Tesarik *et al.*, 2002). Another study proposed that the ability of an oocyte to respond to sperm-induced calcium oscillations is dependent on the presence of machinery in the oocyte that is functional only once in mammalian oocytes, and is activated fully by sperm components but not after parthenogenetic activation (Cheung *et al.*, 2000; Alberio *et al.*, 2001). They demonstrated that the failure of oocytes to generate Ca^{2+} transients in response to sperm may be one of the contributing factors to the poor rates of development in embryos from 19 day old mouse oocyte donors compared to 24 day old donors. These data indicated that changes occur in growing oocytes that modify their capacity for releasing Ca^{2+}. The ability to generate Ca^{2+} transients in response to sperm is initiated during oocyte growth and continues through oocyte maturation. We showed that AOA can overcome this apparent lack of maternal modifications necessary to generate sufficient Ca^{2+} oscillations in response to sperm.

Micromanipulation-driven Ca^{2+} activation is a new modified micromanipulation technique using vigorous aspiration of oocyte cytoplasm and may offer a useful alternative for

Ca ionophores (Tesarik *et al.*, 2002). The clinical use of ionophores in assisted reproduction is limited by insufficient knowledge about their potential toxic effect on oocytes and embryos. However, preimplantation development seemed not to be affected by ionophore treatment in our study.

In conclusion, we verified whether heterologous mouse ICSI can act as a reliable diagnostic tool when investigating fertilization failure after ICSI. The sperm-borne oocyte deficiency was easily shown by the failure of mouse oocyte activation by the patient's sperm. When the MOAT shows oocyte-borne deficiencies the interpretation is more difficult and clinical treatment by Ca ionophore treatment may not be so straightforward. In the case of suspected oocyte-related fertilization failure it is advisable to seek confirmation of actual fertilization failure by applying AOA only on part of the oocytes. Finally, we were able to restore fertilization rates and to obtain pregnancies in both sperm- and oocyte-related fertilization deficiency groups. Based on these results we recommend AOA as a reasonable and efficient treatment option in cases of fertilization failure after ICSI. If larger-scale studies can confirm and fine-tune the present findings, AOA may become a standard treatment policy in cases of both oocyte- or sperm-related fertilization failure.

Acknowledgements

References

Alberio R, Zakhartchenko V, Motlik J and Wolf E (2001) Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer. Int J Dev Biol 45,797–809.

Battaglia DE, Koehler JK, Klein NA and Tucker MJ (1997) Failure of oocyte activation after intracytoplasmic sperm injection using round-headed sperm. Fertil Steril 68,118–122.

Ben-Yosef D and Shalgi R (2001) Oocyte activation: lessons from human infertility. Trends Mol Med 7,163–169.

Cheung A, Swann K and Carroll J (2000) The ability to generate normal Ca^{2+} transients in response to spermatozoa develops during the final stages of oocyte growth and maturation. Hum Reprod 15,1389–1395.

Chi HJ, Koo JJ, Song SJ, Lee JY and Chang SS (2004) Successful fertilization and pregnancy after intracytoplasmic sperm injection and oocyte activation with calcium ionophore in a normozoospermic patient with extremely low fertilization rates in intracytoplasmic sperm injection cycles. Fertil Steril 82,475–477.

Dozortsev D, Qian C, Ermilov A, Rybouchkin A, De Sutter P and Dhont M (1997) Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of the sperm–oocyte interaction. Hum Reprod 12,2792–2796.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S and Ozil JP (2002) Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. Dev Biol 15,280–291.

Ebner T, Moser M, Sommergruber M, Jesacher K and Tews G (2004) Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. Hum Reprod 19,1837–1841.

Eldar-Geva T, Brooks B, Margalioth EJ, Zylber-Haran E, Gal M and Silber SJ (2003) Successful pregnancy and delivery after calcium ionophore oocyte activation in a normozoospermic patient with previous repeated failed fertilization after intracytoplasmic sperm injection. Fertil Steril 79 (Suppl 3),1656–1658.

Kim ST, Cha YB, Park JM and Gye MC (2001) Successful pregnancy and delivery from frozen–thawed embryos after intracytoplasmic sperm injection using round-headed spermatozoa and assisted oocyte activation in

a globozoospermic patient with mosaic Down syndrome. Fertil Steril 75, 445–447.

Kovacic B and Vlaisavljevic V (2000) Configuration of maternal and paternal chromatin and pertaining microtubules in human oocytes failing to fertilize after intracytoplasmic sperm injection. Mol Reprod Dev 55, 197–204.

Lawitts JA and Biggers JD (1991) Optimization of mouse embryo culture media using simplex methods. J Reprod Fertil 91,543–556.

Mahutte NG and Arici A (2003) Failed fertilization: is it predictable? Curr Opin Obstet Gynecol 15,211–218.

Murase Y, Araki Y, Mizuno S, Kawaguchi C, Naito M, Yoshizawa M and Araki Y (2004) Pregnancy following chemical activation of oocytes in a couple with repeated failure of fertilization using ICSI: case report. Hum Reprod 19,1604–1607.

Nakamura S, Terada Y, Horiuchi T, Emuta C, Murakami T, Yaegashi N and Okamura K (2002) Analysis of the human sperm centrosomal function and the oocyte activation ability in a case of globozoospermia, by ICSI into bovine oocytes. Hum Reprod 17,2930–2934.

Rybouchkin A, Dozortsev D, Pelinck MJ, De Sutter P and Dhont M (1996) Analysis of the oocyte activation capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. Hum Reprod 11,2170–2175.

Rybouchkin AV, Van der Straeten F, Quatacker J, De Sutter P and Dhont M (1997) Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. Fertil Steril 68,1144–1147.

Sousa M and Tesarik J (1994) Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. Hum Reprod 9,2374–2380.

Stone S, O'Mahony F, Khalaf Y, Taylor A and Braude P (2000) A normal livebirth after intracytoplasmic sperm injection for globozoospermia without assisted oocyte activation: case report. Hum Reprod 15,139–141.

Tesarik J and Sousa M (1995) More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. Fertil Steril 63,343–349.

Tesarik J and Mendoza C (1999) In vitro fertilization by intracytoplasmic sperm injection. Bioessays 21,791–801.

Tesarik J, Mendoza C and Greco E (2000) The activity (calcium oscillator?) responsible for human oocyte activation after injection with round spermatids is associated with spermatid nuclei. Fertil Steril 74,1245–1247.

Tesarik J, Rienzi L, Ubaldi F, Mendoza C and Greco E (2002) Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne oocyte activation failures. Fertil Steril 78,619–624.

Yanagida K, Katayose H, Yazawa H, Kimura Y, Sato A, Yanagimachi H and Yanagimachi R (1999) Successful fertilization and pregnancy following ICSI and electrical oocyte activation. Hum Reprod 14,1307–1311.

Yanagida K, Katayose H, Hirata S, Yazawa H, Hayashi S and Sato A (2001) Influence of sperm immobilization on onset of Ca(2 +) oscillations after ICSI. Hum Reprod 16,148–152.