ISSN: 2047-2919

ORIGINAL ARTICLE

Correspondence:
Edson Borges, Fertility – Centro de Fertilização Assistida, Av. Brigadeiro Luis Antônio 4545, São Paulo 01401-002, Brazil. E-mail: edson@fertility.com.br

Keywords:
azoospermia, intracytoplasmic sperm injection, sperm retrieval

Received: 21-Mar-2013
Revised: 4-Jun-2013
Accepted: 5-Jun-2013

doi: 10.1111/j.2047-2927.2013.00112.x

The male factor of infertility should not be an issue for the selection of patients for extended embryo culture programmes

[1,2]D. P. A. F. Braga, [1,2]A. S. Setti, [1]L. Vingris, [1]R. C. S. Figueira, [1]A. Iaconelli and [1,2]E. Borges

[1]Fertility – Centro de Fertilização Assistida, São Paulo, Brazil, and [2]Instituto Sapientiae – Centro de Estudos e Pesquisa em Reprodução Humana Assistida, São Paulo, Brazil

SUMMARY

This study evaluated the influence of sperm origin and basic sperm parameters on blastocyst implantation competence. The study included 2912 embryos obtained from 370 patients undergoing intracytoplasmic sperm injection cycles, with embryo transfer on day 5 of development. The embryos were divided into experimental groups according to their origin: (i) embryos originated from ejaculated-derived spermatozoa (Ejaculated group, $n = 2093$), from epididymal-derived spermatozoa (Epididymal group, $n = 463$) and from testicular-derived spermatozoa (Testicular group, $n = 356$). The groups were compared in relation to their blastocyst implantation competence. In addition, the influence of sperm parameters on blastocyst implantation was investigated. The sperm origin was determinant to the success of implantation. When blastocysts originating from testicle-derived spermatozoa were transferred, 66.4% implanted, while only 35.8 and 48.6% of blastocysts originated from epididymis- and ejaculate-derived spermatozoa implanted respectively ($p = 0.001$). The sperm volume and concentration were increased in cycles in which the implantation rate was 100 compared to the 0% implantation rate cases; however, the sperm motility and morphology did not differ among the groups. These results suggest that, with the exception of sperm volume and concentration, the male factor of infertility should not be an issue for the selection of patients for extended embryo culture programmes, even when azoospermic patients are considered.

INTRODUCTION

Extended embryo culture and the subsequent transfer of blastocyst stage embryos are associated with increased implantation rates when compared with cleavage stage embryo transfers (Blake *et al.*, 2007; Papanikolaou *et al.*, 2008). Prolonging the culture period allows for a better selection of embryos with a higher implantation potential and a better synchronization between the endometrium and the embryo. Moreover, because of their high implantation rate, single blastocysts transfers may increase pregnancy rates and reduce multiple gestations (Gardner *et al.*, 2004; Ryan *et al.*, 2007).

Although several studies have shown an improved outcome from blastocyst transfer, not all patients benefit from postponing the embryo transfer. Several clinical factors have been described as being associated with the development of low quality blastocysts after an extended embryo culture (Thomas *et al.*, 2010). The relationships between blastocyst developmental competence and maternal age (Janny & Menezo, 1996; Porter *et al.*, 2002; Shapiro *et al.*, 2002), method of insemination (Dumoulin *et al.*, 2000; Thomas *et al.*, 2010), semen quality (Miller & Smith, 2001; Seli *et al.*, 2004; Vanderzwalmen *et al.*, 2008), cause of infertility (Hsieh *et al.*, 2000) and cleavage stage embryo quality (Neuber *et al.*, 2003; Guerif *et al.*, 2010) have been reported. However, less is known about the effect of sperm origin on blastocyst developmental capacity.

Since the first report of a birth after intracytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992), this technique has been used specially to treat severe male infertility. In addition to ejaculated spermatozoa, testicular and epididymal spermatozoa can be used for ICSI (Craft *et al.*, 1993; Schoysman *et al.*, 1993) with high levels of fertilization and good pregnancy rates (Dohle *et al.*, 1998; Palermo *et al.*, 1999).

Although the success rates of ICSI were thought to be independent of the basic sperm parameters (Kupker *et al.*, 1995; Nagy *et al.*, 1995), recent reports have suggested that repeated failures after ICSI may arise from spermatozoa-derived factors on preimplantation embryo development (Tesarik, 2005; Tesarik *et al.*, 2006). The effect of the sperm source on ICSI outcome has been widely discussed. We have previously reported that, in

azoospermic patients, embryo quality depends on the origin of the injected spermatozoa (Rossi-Ferragut *et al.*, 2003). Pasqualotto *et al.* (2002) also found that testicular sperm injection resulted in lower fertilization and pregnancy rates when compared with epididymal sperm injection.

However, whether sperm origin affects blastocyst quality and implantation potential is still under debate. Therefore, the goal of this study was to evaluate the influence of sperm origin and basic sperm parameters on the blastocyst implantation competence. In addition, the influence of blastocyst developmental status on the implantation capacity was investigated.

MATERIALS AND METHODS

Study design

This is a retrospective cohort study, performed between January 2011 and July 2012, that included 2912 embryos obtained from 370 patients undergoing ICSI cycles, for the first time. All of the embryos were evaluated at 16–18 h post-ICSI and on days 2, 3 and 5 of development and embryo transfer was performed on day 5 of development.

The embryos were divided into three experimental groups according to their origin: (i) embryos originated from ejaculated-derived spermatozoa (Ejaculated group, $n = 2093$), embryos originated from epididymal-derived spermatozoa (Epididymal group, $n = 463$) and embryos originated from testicular-derived spermatozoa (Testicular group, $n = 356$). The developmental status of the embryos was graded according to Gardner & Schoolcraft (1999) (a modified system).

In second analyses only cycles in which the implantation rate was either 100% or 0% were analysed ($n = 729$) and the sperm origin groups were compared in relation to the implantation (positive implantation vs. negative implantation).

For ejaculate spermatozoa, the influence of sperm parameters (concentration, morphology and motility) on the embryo implantation capacity was also investigated. For this analysis, embryos were divided into two groups: positive and negative implantation groups. The groups were compared in relation to their sperm concentration, sperm motility and sperm morphology.

Written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local institutional review board.

Controlled ovarian stimulation

Controlled ovarian stimulation was achieved using recombinant FSH (Gonal-F; Serono, Geneva, Switzerland), and pituitary blockage was performed using a GnRH antagonist (Cetrotide; Serono).

Follicular growth was monitored by a transvaginal ultrasound examination starting on day 4 of gonadotropin administration. When adequate follicular growth and serum E2 levels were observed, recombinant human chorionic gonadotropin (hCG, Ovidrel; Serono) was administered to trigger the final follicular maturation. Oocytes were collected 35 h after hCG administration by transvaginal ultrasound ovum pick-up.

The recovered oocytes were assessed for their nuclear status, and those in metaphase II were submitted to ICSI following routine procedures (Palermo *et al.*, 1992).

Preparation of oocytes

Retrieved oocytes were maintained in culture media (Global for fertilisation; LifeGlobal, Connecticut, CT, USA), supplemented with 10% protein supplement (LGPS; LifeGlobal) and covered with paraffin oil (Paraffin oil P.G.; LifeGlobal) for 2–3 h before removal of cumulus cells. The surrounding cumulus cells were removed after exposure to a HEPES-buffered medium containing hyaluronidase (80 IU/mL; LifeGlobal). The remaining cumulus cells were mechanically removed by gentle pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA, USA).

Oocyte morphology was assessed using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a Hoffmann modulation contrast system under 400× magnification just prior to sperm injection (4 h after retrieval). The following oocyte dysmorphisms were recorded: (i) cytoplasmic granularity, (ii) cytoplasmic colour, (iii) vacuoles in the ooplasm, (iv), aggregates of smooth endoplasmic reticulum clusters in the ooplasm, (v) a large perivitelline space (PVS), (vi) PVS granularity, (vii) a fragmented polar body (PB), (viii) zona pellucida abnormalities and (ix) oocyte shape abnormalities. Oocytes that were observed to have released the first PB were considered mature and were used for ICSI.

Semen sample collection and preparation

Ejaculated spermatozoa

Semen samples were collected by masturbation after 2–7 days of ejaculatory abstinence. After liquefaction for 30 min at room temperature, the semen samples were evaluated according to the threshold values established by the WHO in 2010 (WHO, 2010). The decision of performing density-gradient centrifugation (DGC) or swim-up was based on semen sample quality. With a suboptimal quality sample a DGC was performed, which is usually preferred for the greater number of mobile spermatozoa selected from poor characteristics samples (low number, motility and morphology samples) (Canale *et al.*, 1994). In addition, the DGC was performed particularly when there was high viscosity semen, elevated leucocytes or high debris contents. For all other sorts of semen samples the method of choice was the swim-up technique.

Epididymal and testicular spermatozoa

After cord block anaesthesia, testicular sperm aspiration (TESA) was performed using a 21-gauge butterfly needle that was longitudinally inserted into the superior testicle pole while avoiding the epididymis. Forward and backward movements and needle direction were changed slightly to ensure parenchymal disruption for needle aspiration.

Percutaneous epididymal sperm aspiration (PESA), under local anaesthesia, was performed using a 27-gauge needle inserted into the epididymis. Gentle, negative pressure was applied as epididymal fluid was aspirated.

For both PESA and TESA, aspirated material was collected into a conical tube and washed with a minimum volume of culture medium at 37 °C. The recovered material was checked for the presence of spermatozoon and centrifuged at 300 ***g*** for 8 min. The fraction was diluted or concentrated if necessary.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was performed in a microinjection dish prepared with 4-μL droplets of buffered medium (Global w/HEPES; LifeGlobal) and covered with paraffin oil on a heated stage (at 37.0 ± 0.5 °C) of an inverted microscope. Approximately 16 h after ICSI, fertilization was confirmed by the presence of two pronuclei and the extrusion of the second PB. Embryos were maintained in a 50-μL drop of culture medium (Global; LifeGlobal), which was supplemented with a 10% protein supplement and covered with paraffin oil, in a humidified atmosphere under 6% CO_2 at 37 °C for 5 days.

Embryo morphology evaluation

Fertilization was assessed at 16–18 h post-ICSI and embryo morphology was assessed on the mornings of days 2, 3 and 5 of embryo development using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon) with a Hoffmann modulation contrast system under 400× magnification.

For the blastocyst developmental status, embryos were given a numerical score from 1 to 6 as follows: 1, an early blastocyst with blastocoels that are less than half of the volume of the embryos; 2, a blastocyst with a blastocoel that is greater than half of the volume of the embryo; 3, a full blastocyst with a blastocoels completely filling the embryo; 4, an expanded blastocyst; 5, a hatching blastocyst; and 6, a hatched blastocyst.

Statistical analyses

The results are expressed as the mean ± SD for numeric variables, while proportions (%) were used for categorical variables.

To calculate the influence of blastocyst developmental status and sperm origin (Ejaculated, Epididymal or Testicular group) on the implantation potential, chi-squared tests were performed.

To calculate the effect of sperm concentration and sperm morphology on the blastocyst implantation group, variance analyses were conducted, while the effect of sperm motility on the implantation group was calculated using a chi-squared test.

In addition, binary regression analysis was used to confirm the influence of the sperm origin on blastocyst implantation status, and the results were expressed as odds ratios (OR), 95% confidence intervals (CI) and p values.

The regression analysis was adjusted for maternal age, the number of retrieved oocytes, endometrial thickness, the total dose of FSH and the fertilization rate, as these parameters could be potential confounders of the association between sperm origin and implantation outcomes.

The results were considered significant at the 5% critical level ($p < 0.05$). Data analysis was carried out using the Minitab Inc. (version 14, State College, PA, USA) Statistical Program.

RESULTS

From 2812 embryos evaluated (ejaculated group, $n = 2093$; epididymal group, $n = 463$ and testicular group, $n = 356$), 1447 (51.4%) reached the blastocyst stage. The developmental status of the blastocysts according to the sperm origin group is described in Table 1.

A total of 729 embryos were transferred on day 5 of embryo development. Most of the transferred embryos (517: 70.8%, 270 patients) were originated from ejaculated-derived spermatozoa, while 120 (16.4%, 59 patients) were originated from epididymis-derived spermatozoa and 92 (12.6%, 41 patients) were originated from testicular-derived spermatozoa. The patient demographic variables are described in Table 2.

Table 1 Blastocyst score according to the sperm origin group

Blastocyst score	Ejaculated group ($n = 2093$)	Epididymal group ($n = 463$)	Testicular group ($n = 356$)
Grade 1	266	61	46
Grade 2	235	42	33
Grade 3	129	25	19
Grade 4	117	24	18
Grade 5	245	49	41
Grade 6	75	14	8

Grade 1: Early blastocysts; Grade 2: Blastocysts with a blastocoel that was greater than half of the volume of the embryo; Grade 3: Full blastocysts; Grader 4: Expanded blastocysts, Grade 5: Hatching blastocysts; and Grade 6: Hatched blastocysts.

Table 2 Patient's demographic characteristics

Variable	Ejaculated group ($n = 270$)	Epididymal group ($n = 49$)	Testicular group ($n = 59$)
Maternal age (year old)	36.7 ± 2.6	38.2 ± 1.7	37.3 ± 3.5
Paternal age (year old)	37.1 ± 7.4	38.8 ± 3.3	37.8 ± 3.5
Total dose of FSH administered (IU)	2191 ± 384	2266 ± 354	2166 ± 386
Number of follicles	9.0 ± 5.3;	10.0 ± 5.4	9.5 ± 6.5
Number of retrieved oocytes	8.4 ± 4.1	9.0 ± 8.8	8.0 ± 7.6
Number of metaphase II oocyte	7.0 ± 2.7	7.0 ± 5.3	6.8 ± 5.3

There were no statistically significant differences among the three groups.

Blastocysts from testicle-derived spermatozoa had the same developmental status as epididymis- and ejaculate-derived spermatozoa (OR: 0.91, CI: 0.78–1.08, $p = 0.276$). However, sperm origin was determinant to the success of implantation. When those blastocysts that originated from testicle-derived spermatozoa were transferred, 66.3% implanted, while only 35.8 and 48.5% of those blastocysts that originated from epididymis- and ejaculate-derived spermatozoa implanted respectively ($p = 0.001$). These data were confirmed by a logistic regression model, which demonstrated a nearly threefold increase in the implantation chance of blastocysts from testicle-derived spermatozoa (OR: 2.82, CI: 1.98–4.08, $p < 0.001$).

The miscarriage rate was not influenced by the origin of the spermatozoa. When those blastocysts that originated from ejaculate-derived spermatozoa were transferred, the miscarriage rate was 14.7%, while the miscarriage rate was 20.9 and 19.6% when blastocysts that originated from epididymis- and testicle-derived spermatozoa were transferred, respectively ($p = 0.543$).

The sperm volume (positive implantation group: 3.21 mL vs. negative implantation group: 2.57 mL, $p < 0.001$) and concentration (positive implantation group: 29.1×10^6/mL vs. negative implantation group: 26.3×10^6/mL, $p < 0.001$) were higher in cycles in which the implantation rate was 100% compared to 0% implantation rate cases; however, the sperm motility (positive implantation group: 51.7 ± 23.0% vs. negative implantation group: 51.3 ± 27.0%, $p = 0.563$) and morphology (positive implantation group: 5.8 ± 5.3% vs. negative implantation group: 6.1 ± 4.7%, $p = 0.453$) did not differ between the groups.

DISCUSSION

Male factor infertility is implicated in approximately 50% of couples treated with assisted reproduction techniques (Maduro & Lamb, 2002), and since its introduction, ICSI has become the treatment of choice for severe male factor infertility. ICSI with non-ejaculated spermatozoa has been performed for more than one decade (Silber *et al.*, 1994) with satisfactory results. However, the impact of using non-ejaculated spermatozoa on the formation of the blastocyst and the implantation competence remains to be elucidated.

Although other studies have reported good embryo development and success rates using different sperm sources, to our knowledge, this is the first manuscript reporting the influence of the sperm origin on blastocyst developmental and implantation competence. This study suggests that the origin of the spermatozoa is determinant to the success of blastocyst implantation. Surprisingly, a nearly threefold increase in the implantation chance of blastocysts from testicle-derived spermatozoa was observed.

Conversely, it has been reported that testicular spermatozoa provides a lower fertilization rate (Pasqualotto *et al.*, 2002) and lower quality embryos (Rossi-Ferragut *et al.*, 2003) when compared to ejaculated and epididymal spermatozoa. Balaban *et al.* (2001) suggested that ICSI with spermatozoa retrieved from testes leads to lower blastocyst formation and implantation. On the other hand, in a previous study by Nilsson *et al.*(2007), it was described that a single blastocyst transfer using epididymal or testicular spermatozoa yields results similar to those of ejaculate ICSI because of oligozoospermia. In addition, a higher implantation rate and lower abortion rate were demonstrated when blastocysts derived from testicular spermatozoa were transferred (Virant-Klun *et al.*, 2003). In this study, prolonged culture of embryos after ICSI with testicular spermatozoa did not decrease clinical results in infertile men with azoospermia. Blastocysts had good prognosis for pregnancy, whereas cleavage stage embryos led to lower pregnancy and implantation rates.

Greco *et al.* (2005) suggested that sperm populations recovered directly from the testis might be less affected by DNA damage than ejaculated sperm populations. It has been argued that the DNA damage detected in ejaculated spermatozoa begins after sperm release from Sertoli cells. In fact, it has been shown that after their release from Sertoli cells, spermatids and spermatozoa appear to suffer DNA damage independently of the usual cell death signalling pathways (Tesarik *et al.*, 2004a,b).

It has been suggested that oxidative stress can be responsible for sperm DNA damage (Buttke & Sandstrom, 1994; Halliwell, 1994, 1996; Agarwal *et al.*, 2003; Moustafa *et al.*, 2004), and the loss of nutritional support by Sertoli cells may aggravate the impact of oxidative stress on sperm cell components (Tesarik *et al.*, 2004a,b).

A previous study showed an improvement in the clinical pregnancy and implantation rate after ICSI with the use of testicular spermatozoa; however, the fertilization rate and embryo morphology were similar when the use of ejaculated or testicular spermatozoa was compared (Greco *et al.*, 2005). This result is in agreement with our findings, which demonstrated that blastocysts originating from testicle-derived spermatozoa have the same developmental competence as blastocysts originating from ejaculated- and testicular-derived spermatozoa; however, the implantation potential is significantly higher when testicular spermatozoa was used. It may be argued that the use of testicular spermatozoa can compensate for the reproductive disadvantage associated with the use of ejaculated spermatozoa for ICSI.

It has been reported that spermatozoa with DNA damage can still fertilize oocytes and give rise to embryos with good morphological appearances, although these embryos mostly fail to implant or are miscarried shortly after implantation (Carrell *et al.*, 2003; Henkel *et al.*, 2004; Tesarik *et al.*, 2004a,b). In fact, it is well known that major gene expression in pre-implantation human embryos starts between the four-cell and the eight-cell stage, when the embryonic genome has begun to be expressed (Braude *et al.*, 1988). At this stage, spermatozoa-derived genes that influence embryo viability have also been disrupted; thus, DNA fragmentation is not thought to be directly involved in the events leading to the development of morphological abnormalities at the early stages of embryo development.

Even though a negative relationship has been observed between semen quality and embryo development (Aytoz *et al.*, 1998; Verza & Esteves, 2008), our evidence suggests that blastocyst implantation competence is not related to sperm motility or morphology. This finding is in accordance with a previous report showing that there is no significant influence from either the type or the extent of sperm impairment on the outcome of ICSI (Nagy *et al.*, 1995).

The main drawback of this study is that only cycles in which at least one blastocyst was available for transfer were included; therefore, the detrimental paternal effect on blastocyst formation competence was hidden. The source and maturity of spermatozoa may affect the rate of blastocyst formation. In fact, according to Balaban *et al.* (2001), ICSI with testicular spermatozoa leads to lower blastocyst formation. However, whether these blastocysts lead to lower implantation competence when compared to epididymal or ejaculated spermatozoa is still under debate.

It has been proposed that an extended embryo culture would avoid such negative paternal effects on embryo development after ICSI (Sakkas, 1999). However, once the blastocyst is formed, the embryos derived from testicular spermatozoa may have a good prognosis for pregnancy (Virant-Klun *et al.*, 2003).

In conclusion, the results presented here suggest that, excepting sperm volume and concentration, the male factor of infertility should not be an issue for the selection of patients for extended embryo culture programmes, even when azoospermic patients are considered. Nevertheless, the retrospective nature of the study and lack of a sample size calculation limit its conclusions, therefore more studies should be performed to confirm our findings.

REFERENCES

Agarwal A, Saleh RA & Bedaiwy MA. (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 79, 829–843.

Aytoz A, Camus M, Tournaye H, Bonduelle M, Van Steirteghem A & Devroey P. (1998) Outcome of pregnancies after intracytoplasmic sperm injection and the effect of sperm origin and quality on this outcome. *Fertil Steril* 70, 500–505.

Balaban B, Urman B, Isiklar A, Alatas C, Mercan R, Aksoy S *et al.* (2001) Blastocyst transfer following intracytoplasmic injection of ejaculated, epididymal or testicular spermatozoa. *Hum Reprod* 16, 125–129.

Blake DA, Farquhar CM, Johnson N & Proctor M. (2007) Cleavage stage versus blastocyst stage embryo transfer in assisted conception. *Cochrane Database Syst Rev* 17, CD002118.

Braude P, Bolton V & Moore S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332, 459–461.

Buttke TM & Sandstrom PA. (1994) Oxidative stress as a mediator of apoptosis. *Immunol Today* 15, 7–10.

Canale D, Giorgi PM, Gasperini M, Pucci E, Barletta D, Gasperi M, et al. (1994) Inter and intra-individual variability of sperm morphology after selection with three different techniques: layering, swimup from pellet and percoll. *J Endocrinol Invest* 17, 729–732.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L *et al.* (2003) Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* 49, 49–55.

Canale D, Giorgi PM, Gasperini M, Pucci E, Barletta D, Gasperi M *et al.* (1994) Inter and intra-individual variability of sperm morphology after selection with three different techniques: layering, swimup from pellet and percoll. *J Endocrinol Invest* 17, 729–732.

Craft I, Bennett V & Nicholson N. (1993) Fertilising ability of testicular spermatozoa. *Lancet* 342, 864.

Dohle GR, Ramos L, Pieters MH, Braat DD & Weber RF. (1998) Surgical sperm retrieval and intracytoplasmic sperm injection as treatment of obstructive azoospermia. *Hum Reprod* 13, 620–623.

Dumoulin JC, Coonen E, Bras M, van Wissen LC, Ignoul-Vanvuchelen R, Bergers-Jansen JM *et al.* (2000) Comparison of in-vitro development of embryos originating from either conventional in-vitro fertilization or intracytoplasmic sperm injection. *Hum Reprod* 15, 402–409.

Gardner D & Schoolcraft W. (1999) In-vitro culture of human blastocysts. In: *Towards Reproductive Certainty: Fertility and Genetics Beyond 1999* (eds. R Jansen & D Mortimer), pp. 378–388. Parthenon Press, Carnforth, UK.

Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J & Schoolcraft WB. (2004) Single blastocyst transfer: a prospective randomized trial. *Fertil Steril* 81, 551–555.

Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S *et al.* (2005) Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Hum Reprod* 20, 226–230.

Guerif F, Lemseffer M, Leger J, Bidault R, Cadoret V, Chavez C *et al.* (2010) Does early morphology provide additional selection power to blastocyst selection for transfer? *Reprod Biomed Online* 21, 510–519.

Halliwell B. (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* 344, 721–724.

Halliwell B. (1996) Free radicals, proteins and DNA: oxidative damage versus redox regulation. *Biochem Soc Trans* 24, 1023–1027.

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R *et al.* (2004) Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 81, 965–972.

Hsieh YY, Tsai HD & Chang FC. (2000) Routine blastocyst culture and transfer: 201 patients' experience. *J Assist Reprod Genet* 17, 405–408.

Janny L & Menezo YJ. (1996) Maternal age effect on early human embryonic development and blastocyst formation. *Mol Reprod Dev* 45, 31–37.

Kupker W, al-Hasani S, Schulze W, Kuhnel W, Schill T, Felberbaum R & Diedrich K. (1995) Morphology in intracytoplasmic sperm injection: preliminary results. *J Assist Reprod Genet* 12, 620–626.

Maduro MR & Lamb DJ. (2002) Understanding new genetics of male infertility. *J Urol* 168, 2197–2205.

Miller JE & Smith TT. (2001) The effect of intracytoplasmic sperm injection and semen parameters on blastocyst development in vitro. *Hum Reprod* 16, 918–924.

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

Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M *et al.* (1995) The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum Reprod* 10, 1123–1129.

Neuber E, Rinaudo P, Trimarchi JR & Sakkas D. (2003) Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development. *Hum Reprod* 18, 1307–1312.

Nilsson S, Waldenstrom U, Engstrom AB & Hellberg D. (2007) Single blastocyst transfer after ICSI from ejaculate spermatozoa, percutaneous epididymal sperm aspiration (PESA) or testicular sperm extraction (TESE). *J Assist Reprod Genet* 24, 167–171.

Palermo G, Joris H, Devroey P & Van Steirteghem AC. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340, 17–18.

Palermo GD, Schlegel PN, Hariprashad JJ, Ergun B, Mielnik A, Zaninovic N *et al.* (1999) Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. *Hum Reprod* 14, 741–748.

Papanikolaou EG, Kolibianakis EM, Tournaye H, Venetis CA, Fatemi H, Tarlatzis B *et al.* (2008) Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. *Hum Reprod* 23, 91–99.

Pasqualotto FF, Rossi-Ferragut LM, Rocha CC, Iaconelli A, Jr & Borges E, Jr. (2002) Outcome of in vitro fertilization and intracytoplasmic injection of epididymal and testicular sperm obtained from patients with obstructive and nonobstructive azoospermia. *J Urol* 167, 1753–1756.

Porter RN, Tucker MJ, Graham J & Sills ES. (2002) Advanced embryo development during extended in vitro culture: observations of formation and hatching patterns in non-transferred human blastocysts. *Hum Fertil (Camb)* 5, 215–220.

Rossi-Ferragut LM, Iaconelli A, Jr, Aoki T, Rocha CC, dos Santos DR, Pasqualotto FF *et al.* (2003) Pronuclear and morphological features as a cumulative score to select embryos in ICSI (intracytoplasmic sperm injection) cycles according to sperm origin. *J Assist Reprod Genet* 20, 1–7.

Ryan GL, Sparks AE, Sipe CS, Syrop CH, Dokras A & Van Voorhis BJ. (2007) A mandatory single blastocyst transfer policy with educational campaign in a United States IVF program reduces multiple gestation rates without sacrificing pregnancy rates. *Fertil Steril* 88, 354–360.

Sakkas D. (1999) The use of blastocyst culture to avoid inheritance of an abnormal paternal genome after ICSI. *Hum Reprod* 14, 4–5.

Schoysman R, Vanderzwalmen P, Nijs M, Segal L, Segal-Bertin G, Geerts L *et al.* (1993) Pregnancy after fertilisation with human testicular spermatozoa. *Lancet* 342, 1237.

Seli E, Gardner DK, Schoolcraft WB, Moffatt O & Sakkas D. (2004) Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril* 82, 378–383.

Shapiro BS, Richter KS, Harris DC & Daneshmand ST. (2002) Influence of patient age on the growth and transfer of blastocyst-stage embryos. *Fertil Steril* 77, 700–705.

Silber SJ, Nagy ZP, Liu J, Godoy H, Devroey P & Van Steirteghem AC. (1994) Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. *Hum Reprod* 9, 1705–1709.

Tesarik J. (2005) Paternal effects on cell division in the human preimplantation embryo. *Reprod Biomed Online* 10, 370–375.

Tesarik J, Greco E & Mendoza C. (2004a) Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 19, 611–615.

Tesarik J, Ubaldi F, Rienzi L, Martinez F, Iacobelli M, Mendoza C *et al.* (2004b) Caspase-dependent and -independent DNA fragmentation in Sertoli and germ cells from men with primary testicular failure: relationship with histological diagnosis. *Hum Reprod* 19, 254–261.

Tesarik J, Mendoza-Tesarik R & Mendoza C. (2006) Sperm nuclear DNA damage: update on the mechanism, diagnosis and treatment. *Reprod Biomed Online* 12, 715–721.

Thomas MR, Sparks AE, Ryan GL & Van Voorhis BJ. (2010) Clinical predictors of human blastocyst formation and pregnancy after extended embryo culture and transfer. *Fertil Steril* 94, 543–548.

Vanderzwalmen P, Hiemer A, Rubner P, Bach M, Neyer A, Stecher A *et al.* (2008) Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. *Reprod Biomed Online* 17, 617–627.

Verza S, Jr & Esteves SC. (2008) Sperm defect severity rather than sperm source is associated with lower fertilization rates after intracytoplasmic sperm injection. *Int Braz J Urol* 34, 49–56.

Virant-Klun I, Tomazevic T, Zorn B, Bacer-Kermavner L, Mivsek J & Meden-Vrtovec H. (2003) Blastocyst formation–good indicator of clinical results after ICSI with testicular spermatozoa. *Hum Reprod* 18, 1070–1076.

WHO (2010) *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. World Health Organization, Geneva.

