Embryo Transfer Techniques and Their Impact on The Outcome of Assisted Conception

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SUMMARY

This thesis describes the development of a novel system to grade the 'difficulty' of an embryo transfer (ET) procedure. A systematic review of the prevalence of 'difficult' ET has revealed marked heterogeneity across the literature with no real consensus 'definition' or 'grading system' available for clinicians to use. A new systematic approach to grading ET procedures has been proposed and tested. It has the ablity to predict IVF outcomes accurately and has proven to be reliable.

The 'difficult' ET procedure has been shown in this thesis to be detrimental to IVF outcomes when a fresh single blastocyst is transferred. All embryos of different qualities are affected to some degree by a difficult ET, but it appears that high quality blastocysts are proportionally more affected than the lower quality embryos, which have a lower potential to implant, though the mechanism is not clear.

The embryo transfer time (ETT) is fundamental in determining the implantation potential of a single blastocyst. Once the ET procedures takes longer than '120-seconds' and ETT is prolonged, there is a significant impact on both pregnancy and clinical pregnancy rates. The detrimental impact of increasing ETT on pregnancy rates was found to be independent of an embryo's quality. The ETT appears to be a more accurate way to predict IVF outcomes than a stratified grading scale, though combining a descriptive scale with ETT may offer the optimum grading system.

This thesis has shown that embryo cooling during ET loading and discharging may be significant. The morphokinetic data evaluated in developing mouse embryos has suggested that embryo hatching may be affected by embryo cooling, but further work is needed to confirm this effect. The 'novel' grading system proposed here combines both ETT with a systematic grading system and is based on strong evidence acquired in this work. It has been shown to be reliable and easy to use but it remains for this 'new' system to be tested and integrated into clinical practice.

ABBREVIATIONS

ART	Assisted Reproductive Technology
cm	centimetre
CO_2	Carbon dioxide
CPR	Clinical Pregnancy Rate
CI	Confidence Interval
°C	Degrees centigrade
EP	Ectopic Pregnancy
ET	Embryo Transfer
ETT	Embryo Transfer Time
ICM	Inner cell mass
ICSI	Intracytoplasmic Sperm Injection
PR	Pregnancy Rate
IVF	In vitro Fertilisation
LB	Live birth rate
Mins	Minutes
MET	Mock Embryo Transfer
PBS	Phosphate buffered solution
PVA	Paraformaldehyde
RCT	Randomised Controlled Trial
RT	Room temperature
Secs	Seconds
UCA	Utero-cervical Angle
ZP	Zona pellucida

DEFINITIONS

Pregnancy rate

The pregnancy rate was defined as a positive urinary pregnancy test on day 13 following single embryo transfer.

Implantation rate

The implantation rate was defined as the number of gestational sacs observed on ultrasound at 6 weeks of pregnancy divided by the number of embryos transferred.

Clinical pregnancy rate

The clinical pregnancy rate was defined as the number of fetal heartbeats seen on ultrasound at six weeks' gestation

Live birth rate

The live birth rate was defined as the birth of a live baby after 24 weeks' gestation.

DECLARATION OF ORIGINALITY

This thesis constitutes original material, except where indicated in the text for illustrations and background material recreated from referenced sources. This thesis is my own work, with specific contributions as now described.

All human data series in this thesis were collected from patients undergoing NHS or privately funded IVF cycles at Nurture Fertility, Nottingham UK. IVF outcomes and observations recorded in no way altered the course of any patient's treatment. Animal data was collated in Assisted Reproduction Laboratories at the University of Nottingham. Post-doctoral research technician: Lessly Sepulveda Ricon assisted with the animal laboratory work and with the handling of mouse embryos. Sonia Asif assisted with abstract screening in the systematic review. I collated, analysed and interpreted all embryo transfer data sets myself. Dr Andrea Venn, an Honorary Associate Professor and Statistician at the University of Nottingham assisted with the statistical planning. Mr Nicholas Raine-Fenning (Clinical Associate Professor & Reader in Reproductive Medicine and Surgery), Dr Walid Maalouf (Assistant Professor in Embryology) and Mr James Hopkisson, (Associate Professor in Reproductive Medicine and Surgery) provided clinical supervision and academic advice.

I certify that this is a true account of my contribution to the work presented in this thesis and it has not been previously accepted for a higher degree.

Signature of Candidate…….. Date……

ACKNOWLEDGEMENTS

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Chapter 1

Background & Hypotheses

1.1 BACKGROUND

Assisted conception techniques have advanced significantly since the birth of the first successful human in-vitro fertilisation (IVF) baby. Louise Brown was delivered on 25th July 1978 by caesarean section and proved that new life could be created using assisted conception techniques. This landmark birth signalled a new era for reproductive medicine and assisted conception techniques.

It was the culmination of 10 years' work for Robert Edwards and Patrick Steptoe, who demonstrated beautifully how the collaboration of science and clinical medicine can achieve dramatic results (Steptoe and Edwards, 1978). There are now well over six million babies born by IVF in the world today.

Almost forty years after that first successful assisted conception in the laboratory there has been an explosion of demand from sub-fertile patients requiring assisted reproductive technologies (ART). Embryos created in a laboratory under controlled conditions are now replaced in the uterus of sub-fertile women every day.

There are several techniques used in the creation of human embryos with IVF. Once the embryos have been cultured in the laboratory in an incubator the critical last step is to replace the embryo in the uterus. This relatively simple technique has been the subject of rigorous debate over the years yet remains relatively unchanged.

1.2 EMBRYO TRANSFER & HISTORY

1.2.1 Embryo Transfer – What is it?

Embryo Transfer (ET) is the final critical step undertaken in an IVF treatment cycle. ET refers to the clinical technique used to replace an embryo cultured in an incubator under laboratory conditions back into the uterus of a patient. It can be a relatively straight forward procedure to perform but requires a skilled clinician.

The ET procedure starts with a sterile speculum being placed in the vagina and a full 360-degree view of the cervix is obtained. The cervix is cleaned and cervical mucus is often aspirated or removed prior to embryo transfer. A catheter is loaded with the embryo between two air bubbles and then carefully threaded through the cervical canal and into the uterus. Here the embryo is deposited and then the catheter is withdrawn and the embryologist checks the catheter to ensure it is empty. The next stage is to wait to see if implantation is successful with a positive urine pregnancy test two weeks later.

1.1.1 Embryo Transfer - History

The concept that a baby might be transferred from one woman's body to another was first depicted in a painting of the birth of Mahavira in 599 BC (). Saints and founders of religions had to be born by royalty and so Mahavira, the founder of the

Jain religion, is shown being implanted into Queen Trisala's uterus after initially being conceived in Devananda's body. The transfer was undertaken by the horse-headed creature, Harinegameshin (Betteridge, 2003). Almost two thousand five hundred years later the first actual human ET took place.

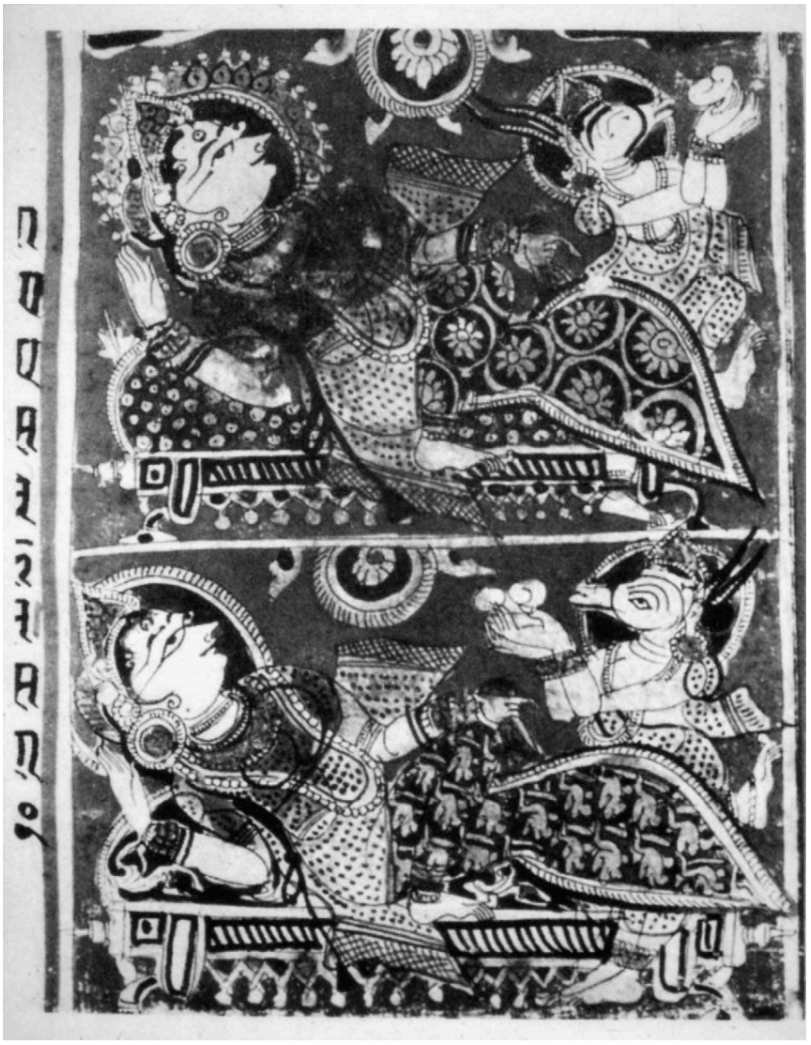


Figure 1.1.1-1 A painting depicting the birth of Mahavira, the founder of the Jain religion, in 599 B.C. Devananda sleeping at the top, is the wife of a priest, in whom the Mahavira was conceived. It was transplanted into the womb of Queen Trisala, sleeping at the bottom by the horse-headed creature, Harinegameshin. The transfer was necessary as founders of religions must be born of royalty (Guttman, 1980).

Much success in human IVF owes its roots to research undertaken in animal reproductive biology. In the late 19th Century it was Walter Heape (1855-1929) though who first successfully produced live young rabbits following embryo transfer. Heape undertook the first ET of fertilised rabbit ova in a laboratory in his home in Prestwich, Manchester with the assistance of a well-known surgeon Mr Samuel Buckley. The birth of these rabbits showed it to be possible to transfer the embryos of one rabbit into the uterus of another with no deleterious effect.

Robert Edwards was a scientist from Manchester and together with Patrick Steptoe a gynaecologist from Oldham they were the first to successfully transfer a human blastocyst into a sub fertile woman's uterus and create a viable fetus.

1.1 IMPORTANCE OF EMBRYO TRANSFER TO IVF

IVF outcomes depend on several factors; including ovarian stimulation protocols, culture media, laboratory conditions, endometrial receptivity, embryo quality and the ET technique (Roseboom et al., 1995). These factors impact not just on the positive outcomes of IVF treatment such as clinical pregnancy and live birth rates but also the negative outcomes influencing miscarriage and ectopic rates. The ET technique has been studied with varying degrees of consistency and rigour over the years, due to the challenges of testing such a subjective variable. Defining 'difficulty' of ET is a problem that researchers have faced and leads to inconsistency.

1.1.1 Success Rates

Clinical pregnancy and live birth rates vary from one IVF clinic to another and appear to be related to several of these important factors mentioned above. Studies that have looked at how clinical pregnancy and live birth rates change according to ET difficulty have in general agreed that more difficult embryo transfers are detrimental to success (Englert et al., 1986, Mansour et al., 1990, Visser et al., 1993, Wood et al., 2000). Many of these studies are limited by the fact that they have failed to control for embryo quality, which is now known to be an important factor in implantation success (Richardson et al., 2015). Although ET is relatively simple to perform, it seems difficult transfers have been shown to reduce biochemical and clinical pregnancy rates. One large study undertaken in Finland looked at 4807 IVF cycles over a six-year period and it was found that ET difficulty was an independent factor that had a significant effect on implantation and pregnancy rates (Tomas et al., 2002).

1.1.2 Miscarriage

There is a distinct paucity of data looking at the impact that ET seems to have on miscarriage rates either biochemically or clinically. There has been one study in 2008 that looked retrospectively at whether implantation, clinical pregnancy and miscarriage rates were affected when ET was ultrasound guided. Miscarriage rates were not shown to change significantly with ET technique (Ali et al., 2008). Further

work is needed in this area to really clarify the impact a difficult transfer has on embryos of varying qualities.

1.1.3 Ectopic Pregnancy

Ectopic pregnancy (EP) is a form of abnormal pregnancy where a fertilised ovum implants outside of the uterus. It is estimated that the ectopic pregnancy rate with natural conception is around 1-2%. Ectopic pregnancy related haemorrhage remains one of the commonest reasons for maternal mortality in early pregnancy. The risk of an ectopic pregnancy following assisted reproductive techniques has been shown to range from 2.1%-8.6% in all pregnancies and even reaches as high as 11% in females with tubal factor infertility (Clayton et al., 2006).

It is proposed that the ET technique may be partly responsible for the variable ectopic pregnancy rates because of ART. It has been suggested that uterine contractility and peristalsis increase with ET and can result in reverse migration of embryos out of the uterus increasing the chances of ectopic pregnancy and lower pregnancy rates (Zhu et al., 2014). Another theory is that ectopic pregnancy rates increase because of the hydrostatic pressure forcing the embryo through the tubal ostia during transfer (Azem et al., 1993). There has been much debate about the best place to position the ET catheter and release the embryo to optimise implantation and minimise ectopic pregnancy rates. Tiras et al (2010) argue that this should be in the upper quarter of the uterus and this would appear to be supported

by Rovei et al. 2013 but there is little consensus (Tiras et al., 2010, Rovei et al., 2013).

1.2 EMBRYO TRANSFER FACTORS AFFECTING IVF OUTCOMES

1.2.1 Two-dimensional Ultrasound and Embryo Transfer

Strickler et al. (1985) and Leong et al. (1986) were the first to propose the idea that clinical pregnancy rates might increase with ultrasound-guided ET (Strickler et al., 1985, Leong et al., 1986). Until this point the main ET method was the 'clinical-touch' technique where the clinician would blindly feed the catheter through the cervix and rely on proprioceptive feedback to help them decide whether the catheter tip was in the uterus appropriately. The main disadvantage of the clinical touch method was that the procedure was blinded and so the catheter tip could reach the fundus of the uterus.

Hurley et al (1991) was first to perform a study comparing transvaginal ultrasound-guided embryo transfers to a control group using the clinical touch method (Hurley et al., 1991). They demonstrated that pregnancy rates were higher in the ultrasound-guided cohort, but they only reached statistical significance in a sub-group of single embryo transfers. This lack of significance and conflicting evidence from Al-Shawaf et al (1993) and Kan et al (1999) suggested that using ultrasound for ET did not increase pregnancy rates (al-Shawaf et al., 1993, Kan et al., 1999). This disagreement meant uncertainty amongst the clinicians and so practice did not change immediately.

Despite the clear advantage of seeing the catheter positioned in the uterus and patient satisfaction with this newer technique the 'clinical touch' method persisted and the ultrasound-guided technique was not used for several years.

As the years passed the volume and quality of studies comparing ultrasound guidance to clinical touch grew and they increasingly suggested the ultrasound-guided method offered clear advantage in terms of improving pregnancy rates. A meta-analysis performed by Buckett et al. (2003) looked at a total of eight prospective controlled trials, four of which had been correctly randomised and controlled and these demonstrated a significantly increased chance of clinical pregnancy following ultrasound-guided ET (Buckett, 2003). It is now a well-established concept that ultrasound guidance during ET benefits pregnancy rates.

1.2.2 Three-dimensional Ultrasound

The possibility of 3D ultrasound to improve the success of ET was first proposed by Baba et al. in 2000 (Baba et al., 2000). It was studied in 2005 by Letterie et al. who in a relatively small study of twenty-one patients compared conventional 2D imaging to the 3D images obtained during embryo transfer. This study suggested that there was agreement about catheter tip placement in seventeen of the patients and just four patients' 3D images gave clearer information than would otherwise have been obtained with 2D imaging (Letterie, 2005). There is yet to be a rigorous study showing a real advantage to using 3D ultrasound for embryo transfer.

1.2.3 Embryo Transfer and Bladder

The bladder is anatomically located on the anterior surface of the uterus. It fills and empties regularly. The lower uterus and cervix is adjacent to the bladder and therefore it was proposed by Sundstrom et al (1984) that a change in bladder volume may positively influence the utero-cervical angle (the angle between the uterus and cervical canal) hence straightening the cervical canal and aiding ET (Sundstrom et al., 1984).

No study over the years has successfully tested whether bladder filling has a negative or positive impact on the utero-cervical angle, the ease of embryo transfers and ultimately pregnancy rates. Mitchell et al (1989) attempted to look at this with a randomised controlled trial and showed no significant difference in the difficulty of ET with or without a filled bladder (Mitchell et al., 1989). However, the study was limited as embryo quality and numbers were not considered when looking at the influence of the bladder on ET and implantation rates, so this could be a crucial factor to consider in ET and implantation success.

The studies that have looked at the bladder and its influence on difficulty have always chosen arbitrary volumes of water consumed by test subjects prior to embryo replacement as a measure of whether the bladder is filled or under filled. Lewin et al (1997) strongly criticises this methodology, but then also randomly selects 1000 millilitres (mls) of water to be drunk 60 minutes before embryo transfer to adequately fill the bladder (Lewin et al., 1997). Now that ultrasound

guided embryo transfers are commonplace it would seem far better to assess bladder volume and utero-cervical angle prior to embryo transfer more scientifically. Bladder scanners can be used to accurately estimate the volume of urine in the bladder. It is with this measure and a pre-assessment of utero-cervical angle pre-and post-bladder emptying and mock transfer that will give a more accurate assessment of whether the bladder filling improves the ease of embryo transfer. This research has still not been completed.

1.2.4 Embryo Transfer and Utero-cervical Angle

The ET catheter must pass through the cervix to reach the uterine cavity and one factor that might make this more troublesome is the angle of the uterus and cervix. Uterine version and flexion varies markedly between patients and consequently this affects the utero-cervical angle (UCA). Sallam et al. (2002) gave a concise definition of the 'utero-cervical angle' as being, the 'angle between a line joining the external cervical os and internal cervical os, and a line joining the internal cervical os and uterine fundus' (Figure 1.2.4-1) (Sallam et al., 2002). It is the intersection of these two vectors that is known to be the UCA.

It has been proposed for several years that an acute UCA makes it more difficult to pass an ET catheter, though this was not associated with affecting clinical pregnancy rates (Komaki et al., 2008). This angle is quite variable between patients and possibly impacts on the 'ease' of embryo transfer. In an early study by Johnson et al. (1991) had shown that moderate traction with a tenaculum reduces the utero-cervical

angle considerably making insertion of an intrauterine device easier (Johnson and Bromham, 1991). Although the use of a tenaculum during embryo transfer was common practice for years it was shown to stimulate junctional zone contractions in the uterus, which were considered bad for IVF success (Lesny et al., 1999).

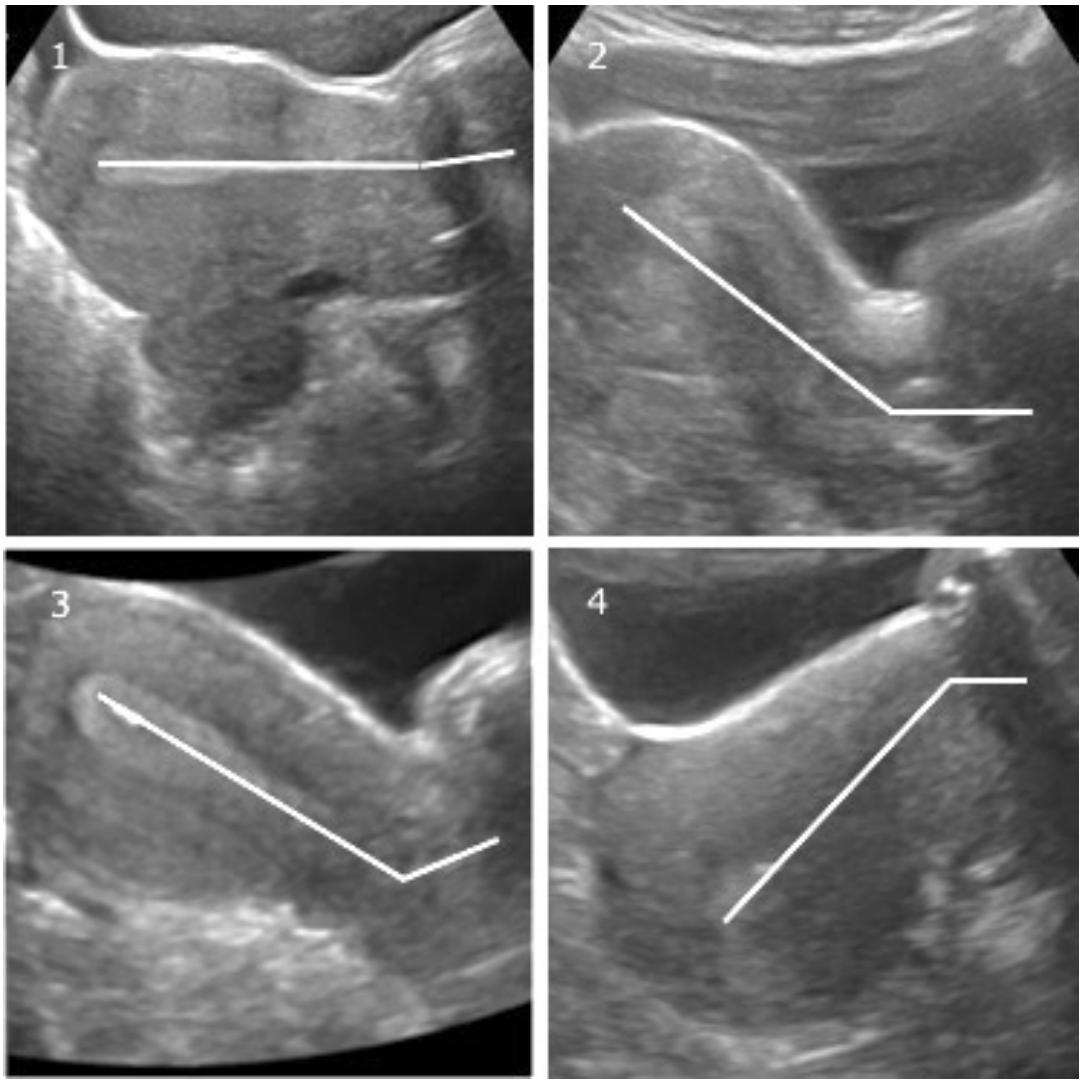


Figure 1.2.4-1 Abdominal ultrasound demonstrating different utero-cervical angles (1) UCA = 0°, (2) UCA > +45°, (3) UCA > +60°, (4) UCA > -45° (Coats, 2017 (in press)).

It is quite possible that patients with an acute UCA may have an easy embryo transfer. One of the major challenges in embryo transfer is to understand and predict which patient will have a difficult ET procedure and use this information to reduce the chances of it occurring. Understanding better the true interaction of utero-cervical angle with IVF success will help to demystify this for clinicians.

1.2.5 Embryo Transfer Catheter Technology

The ET catheter has evolved quickly over the years to meet the demands of a rapidly expanding field of medicine. Due to the challenges of threading a slim catheter through the cervix Ian Craft and his team at The Royal Free in London began looking at materials to find a more malleable embryo transfer catheter in the early 1980's. They were particularly interested in the use of non-toxic malleable plastics rather than metal (Craft et al., 1982). Initial studies in the early 1990's began to consider whether ET catheter selection might affect the success of IVF (Gonen et al., 1991).

Technology and materials have unsurprisingly advanced since 1991, yet at the time an important question was considered as to whether there might be advantages and disadvantages to different catheters. The 'Tom Cat' catheter for example performed much better in difficult embryo transfers and gave higher pregnancy rates than it's close rival the 'Frydman' catheter. These catheters are hardly used today as the IVF market has expanded considerably and with it newer options are available, but the principle of whether an ET catheter itself can affect success rates in IVF remains an important question.

The need to overcome difficult ET with a gradual shift to ultrasound-guided ET, has meant that catheters have evolved and the coaxial catheter and echogenic catheter are now widely used in IVF clinics (Letterie et al., 1999). The two different types of catheter on the market have been the soft and firm catheter systems. Most trials that have been published looking at the outcomes for soft compared with firm catheters have revealed conflicting results. There has been quite a lot of inconsistent methodology in the various studies focused on catheter related IVF success, since not all clinics use the ultrasound-guided ET technique (Meriano et al., 2000, Urman et al., 2000, Karande et al., 2002, McDonald and Norman, 2002, van Weering et al., 2002, Aboulfotouh et al., 2008, Allahbadia et al., 2010b).

Despite these data limitations two meta-analyses were published comparing soft versus firm ET catheters and have associated the soft catheter with higher clinical pregnancy rates, but also showed they were more likely to fail to negotiate the cervix in difficult procedures (Abou-Setta et al., 2005, Buckett, 2006). More recently a study looked specifically at this issue in ultrasound-guided ET patients only and showed no difference in clinical pregnancy rates based on catheter choice (Aboulfotouh et al., 2008).

It is now unusual for ET to not be ultrasound-guided and the catheter usage around the world varies considerably based on the clinician and clinic's preference. This makes it nearly impossible to do genuinely detailed research in this area as practice varies so much. Certainly, the way in which the catheter moves through the cervix

might well be important in terms of distortion and biomechanical stress applied to the tip of the ET catheter. The embryo is loaded into the tip of the catheter and so catheter choice might influence the ease of the ET procedure and consequent effect on the embryo. This is an area of work that needs further investigation to see how catheter choice may alter the direct stress on the embryo depending on the ease of transfer.

1.2.6 Mock Embryo Transfer

Performing a 'mock' embryo transfer ahead of the actual ET procedure is not a new concept. Mansour et al. (1990) looked at this in three-hundred and thirty-five patients and randomised them to either a mock ET prior to the start of IVF treatment to help choose the right catheter, or patients underwent a routine ET on the day with no prior workup. They showed that patients who had a mock transfer had fewer difficult transfers and consequently it was deduced that this improved clinical pregnancy rates (Mansour et al., 1990), though the numbers and quality of embryos compared to standards today differs significantly.

The timing of when is best to do the mock ET has always been a contentious issue. The additional time and cost involved with performing a mock transfer led some clinicians to look at whether it was suitable to perform a mock ET immediately prior to the real ET (Sharif et al., 1995a). It does seem counterintuitive though to stimulate the uterus twice in this way with a mock ET performed so close to the real ET as the risk of over stimulating the uterus may be detrimental. Quite recently this has been

looked at again demonstrating that the mock ET increases the chance of fluid migration with increased uterine contractility and quite possibly also embryo expulsion (Zhu et al., 2014).

Evidence has been slightly conflicting on the issue of 'mock ET timing' though and has demonstrated no detrimental effect on implantation and clinical pregnancy rates when performed close to the day itself for ET (Torre et al., 2010, Katariya et al., 2007). The purpose of the mock transfer is to simulate the conditions of the real ET procedure as close as possible. However, two studies have suggested that with ovarian stimulation not only does the uterine position change particularly in cases of retroversion (Yang et al., 2007) but also the ET depth measured at mock ET changes in almost 60% of cases (Yang et al., 2007, Miller and Frattarelli, 2007). The key question however is whether the mock ET is a good way to predict a difficult transfer. This has never really been assessed fully but seems to be a key fact to establish, and requires a much more uniform and less subjective system to grade difficulty of embryo transfer.

1.2.7 Environmental Conditions and Embryo Transfer Time

Environmental factors have been shown in several studies to have a detrimental impact on embryos (Cohen et al., 1997). Some studies were not specifically looking at IVF embryo transfer procedures but the principles have been extrapolated to this field. Evans et al (1999) looked at 34 gamete intra-fallopian transfer procedures and showed that dependent on the laparoscopic light source there was as significant

difference in pregnancy rates. The Xenon light source emitted significantly more UV-light than the conventional halogen light sources (Evans et al., 1999). Rocha et al. (1998) showed that high environmental temperatures and humidity decrease bovine oocyte quality and the need to control O_2 and CO_2 levels in human embryos and oocytes is well known (Rocha et al., 1998, Bavister, 1995).

Therefore, it has been speculated that once an embryo leaves the incubator for embryo transfer it is much more likely to be exposed to detrimental environmental conditions. The embryo transfer time (ETT) taken from loading the embryo into the transfer catheter to then releasing the embryo in the uterus has been of research interest. Mattoras et al. (2004) looked at 450 cycles of IVF and measured the ETT and showed clinical pregnancy and implantation rates to be much lower with a prolonged ETT >120 seconds. Ultrasound-guided transfer of 3-4 embryos at cleavage stage was undertaken in all cases and ET was graded according to difficulty. Results are somewhat limited by the fact that there was little control for grade of embryos which can significantly affect implantation rates.

A study by Ciray et al (2007) that did control for embryo quality contradicted Matorras' work and showed that in fact the implantation and clinical pregnancy rates were similar with good quality embryos. The poorer quality embryos had a lower implantation and clinical pregnancy rates overall but numbers were insufficient to establish whether implantation rates were significantly lower as the ETT is prolonged. The theory Ciray et al. proposed was that the higher the quality of the oocytes and embryos the more resistant it might be to changes in the

microenvironment, and hence the embryos were able to tolerate prolonged transfers (Ciray et al., 2007). Further investigation is needed to confirm whether the implantation capacity of poorer quality embryos is compromised by a prolonged ETT.

1.2.8 Embryo Transfer Training and Technique

No standard method exists to train clinicians performing embryo transfer yet even though it has been shown to be an important factor affecting ET and IVF success. There have been various attempts to look at the impact of the "physician factor", as one study aptly named it, on IVF success (Karande et al., 1999). Karande et al. (1999) showed that clinical pregnancy rates varied among physicians though they did not look at whether this was influenced by embryo quality differences. Herarns-Stokes (2000) looked more specifically at whether the clinical pregnancy rates varied significantly between 10 clinicians performing 854 embryo transfers, whilst controlling for embryo quality differences (Hearns-Stokes et al., 2000). A clear difference was shown between pregnancy rates for each clinician that could not be explained by embryo quality alone.

The clinician's patience and manual dexterity may be important factors that can influence ET outcomes and hence a training effect was tested by Papageorgiou et al (2001). They looked at five clinical fellows training in the ET technique, and showed three out of the five trainees had a lower pregnancy rate for the first 25 ET procedures compared to the second set of 25 ET procedures they completed

(Papageorgiou et al., 2001). Each trainee had undergone 30 mock ET procedures before starting but the difference identified above suggests a learning curve effect that was still present despite this. It was proposed that perhaps an increased number of mock ET procedures might reduce this initial lower pregnancy rate.

Over the years training in ET techniques has not always been consistent in different programmes and one survey identified that almost half trainees responding were not getting adequate training (Wittenberger et al., 2007). There is clearly a need for quality control of ET procedures and a standard acceptable way to monitor the progress and performance of training new recruits. Various methods can be used to decide if a trainee has reached competency. Most IVF units accept that a trainee completing a pre-specified number of mock transfers is a reliable way to do this. However, Papageorgiou et al. (2001) showed that this 'one-size fits all' model is not guaranteed to work.

A more statistical approach is the cumulative summation test of learning curve (LC-CUSUM) as this is a mathematical way to show when the 'learning curve' (LC) has been achieved by the trainee. The LC-CUSUM was developed to define whether an operator has reached a pre-defined level of performance for a procedure. It is based on what is determined to be acceptable and non-acceptable failure rates with a procedure. Specific to embryo transfer success was determined by a positive urine pregnancy test.

Dessolle et al (2010) attempted to evaluate the LC-CUSUM as a tool to monitor ET training by studying five trainees. LC-CUSUM results revealed that all five trainees reached proficiency by procedure 15 though at varying speeds, and shortly after achieving competence one of the five trainees lost proficiency. It was determined that pregnancy rates dropped significantly when an interval of >10 days existed between ET procedures (Dessolle et al., 2010). Lopez et al (2014) confirmed that the time to reach proficiency varies with individuals and that individualised training with continuous monitoring of the LC of ET is necessary if standards are to be maintained for patients (Lopez et al., 2014).

The next question that remains to be answered is to identify the best method for all clinicians to attain and maintain their competence. A clinician may experience extended periods of time between ET procedures and therefore is there an argument to introduce a simulation training model that will help that clinician return to proficiency more quickly without using the patient as a guinea pig and with LC-CUSUM this should be decided with future research.

1.2.9 Embryo Transfer Catheter Contamination

Contamination of the embryo transfer catheter during procedures has been identified as an important factor that can impact on ET and IVF success rates. The main contaminants that have been considered are microbes, blood and mucus.

Initial work by Egbase et al. (1996) looked at how microbial contamination of the embryo catheter might impact on pregnancy rates (Egbase et al., 1996) and it was initially proposed that microbial contamination might indeed affect pregnancy rates. Two further studies looked at this and showed no clear advantage to prophylactic doxycycline prior to ET (Moore et al., 2000) and whilst upper genital tract contamination with microbes was reduced there was in fact a possible disadvantage to using co-amoxiclav (Peikrishvili et al., 2004) at ET. There was no beneficial impact on pregnancy rates overall and this has more recently been confirmed in a systematic review (Kroon et al., 2012). Alternative antibiotic regimens may still need further investigation but there is no clear advantage to prophylactic antibiotic cover for ET procedures.

Awonuga et al. (1998) established that difficult transfers and reduced pregnancy rates tend to be associated with higher levels of contamination of the ET catheter with mucus and blood (Awonuga et al., 1998a). This was also the conclusion of Goudas et al (1998) who looked specifically at blood contamination and found that blood found outside the transfer catheter not inside it was associated with lower implantation and pregnancy rates (Goudas et al., 1998). Alvero et al (2003) were also interested in this question of blood and mucus contaminating the ET catheter and they completed a retrospective review of 584 ET cycles of high grade embryos transferred under ultrasound-guidance. They showed that for high quality embryos transferred if blood was present on the catheter this was associated with much lower implantation and pregnancy rates (Alvero et al., 2003). A single centre retrospective analysis of 8311 ultrasound guided ET procedures showed a significant

fall in implantation, clinical pregnancy and live birth rates associated with blood on the catheter (Tiras et al., 2012).

However, a systematic review and meta-analysis by Phillips et al (2013) has concluded that the quality of these studies was low and that there is little evidence to suggest that blood on the ET catheter reduces clinical pregnancy rates (Phillips et al., 2013b). It suggested that blood on the catheter is a marker of difficulty rather than being directly responsible for reducing success rates. A recent retrospective study has confirmed with a multivariate analysis that blood on the catheter is not an independent predictor of success and ET 'difficulty' is more predictive of IVF outcomes (Plowden et al., 2017).

Cervical mucus present around the time of ET may contaminate the catheter and affect successful embryo transfer. Visser et al (1993) were the first to suggest that presence of cervical mucus increased the chances of retained embryos following ET and that this was associated with reduced clinical pregnancy rates (Visser et al., 1993). However, Awonuga et al. (1998) found that if catheters were contaminated with mucus and embryos retained then retransferred this did not have an impact on clinical pregnancy rates (Awonuga et al., 1998a). A study by Visschers looked at this issue and randomised a group of patients undergoing ET to cervical cleaning of mucus or not. It found no significant effect of cleaning cervical mucus on success rates and specifically live birth (Visschers et al., 2007). A recent systematic review and meta-analysis of this problem was undertaken by Craciunas et al (2014) and

showed no significant benefit to implantation or live birth rates (Craciunas et al., 2014).

1.2.10 Embryo Transfer and Bed Rest

Historically bed rest was advised for anything from 15 minutes to 24 hours after embryo transfer (Lopata et al., 1980, Edwards et al., 1980). Over the years this issue has been evaluated more scientifically and researchers have shown that bed rest has no beneficial impact on clinical pregnancy rates (Botta and Grudzinskas, 1997, Sharif et al., 1995b). Two randomised studies found that bed rest may be detrimental to implantation rates (Gaikwad et al., 2013, Amarin and Obeidat, 2004). A recent systematic review and meta-analysis of four RCT's of moderate quality including 757 patients was studied by Carucias et al (2016) who confirmed that bed rest is not beneficial following ET. They hypothesised that due to anxiety and stress mechanisms, bed rest may be detrimental to IVF outcomes (Craciunas and Tsampras, 2016).

1.2.11 Bubble Migration and Embryo Transfer

Prior to embryo transfer the embryos are loaded into the ET catheter in the laboratory and sit between two air bubbles. The air bubbles cushion the embryo and hold it securely for transfer through the cervix protecting it from contaminants in the cervix on route into the womb. The change in practice from the 'clinical-touch' method to the 'ultrasound-guided' ET with increasingly more mobile patients after

ET has led to interest in the movement of the air bubbles following transfer, and whether this impacts at all on ET and IVF success.

Woolcott et al. (1998) acknowledge that tracking the air bubbles is somewhat limited in that this is not the same as tracking the embryo but it is the patterns of movement that interested them. Ultrasound performed immediately on standing after ET did not show any significant migration of ET associated air bubbles and supposedly therefore had no impact on embryo position either (Woolcott and Stanger, 1998). Interestingly a few studies that have looked at the position of air bubbles using 2D ultrasound following ET have suggested that the highest pregnancy rates tend to be when the bubbles remain close to the fundus of the uterus (Cenksoy et al., 2014, Friedman et al., 2011, Lambers et al., 2007).

A recent study has looked at this issue with 3D ultrasound and scanned patients at 1, 5 and 60 minutes after ET. This study showed that the embryo-associated air bubbles that remained < 15mm from the fundus around 60 minutes after ET were linked to higher implantation and pregnancy rates (Saravelos et al., 2016). Pregnancy rates were significantly lower if the air bubbles migrated lower in the uterus rather than remaining static or moving towards the fundus. The real challenge is monitoring the position of the embryo that has been transferred, but until there is technology to do this the position and migration of fluid or air bubbles in the uterus is the only option.

1.3 EMBRYO TRANSFER DIFFICULTY

1.3.1 Definition

Measuring the 'difficulty' of an ET is subjective and for this reason it is a challenge to conduct rigorous research in this area of assisted conception. There is currently still no internationally agreed consensus about what defines a 'difficult' embryo transfer.

There are several independent factors that are often considered in the clinician's subjective interpretation of 'difficulty', but there is no uniform description that is applied. Factors such as the time taken to perform an ET, the firmness of catheter needed, the discomfort of the patient, the presence of blood or not and the use of a tenaculum have all been linked to descriptions of difficult ET's. Inter-clinician variability in use of language and interpretation of how difficult a procedure has been makes it very challenging to standardise and grade how difficult the embryo transfer procedures have been.

This presents a considerable challenge in the research field, as without a standardised definition or grading system of difficulty there will always remain bias that is difficult to control for. Most studies that have attempted to grade difficulty have not been especially descriptive but have tended to label the ET as either (1) Easy or (2) Difficult. This basic description is probably unsuitable due to the complex interaction of several factors, already discussed here, related to ET that may affect IVF outcomes.

The lack of a clear definition of ‘difficult’ ET historically means that there are inconsistencies across the literature. Even now studies still use quite different systems to label difficulty, with some reporting a ‘difficult’ ET when both a tenaculum and hysterometer are required (Kava-Braverman et al., 2017), whereas, other studies consider an ET to be difficult if a firmer catheter is required (Singh et al., 2012). There is no clear consensus definition and with a wide variety of ET techniques and ET catheter technology in IVF clinics it becomes impractical to compare results directly in a clinical context. Future research in this area would benefit from a clear definition of ‘difficult’ ET to help move this area of forwards.

A recent study by Ghaffari et al. (2013) defined an easy ET as a ‘smooth procedure that occurred without the use of any force or other instrumentation, without any need to change the catheter (Ghaffari et al., 2013). This in some ways is a slightly simpler way to look at difficult transfer. By defining an easy transfer in this way, it is possible to exclude easy transfers and label all other transfers as difficult. The problem remains that there is no clear standardised grading system or score of difficulty that can be used usefully in a clinical setting and my research should address this.

1.3.2 Prevalence

The lack of a clear definition of ‘difficult’ ET means calculating prevalence is challenging, and the subjectivity involved with reporting it in the first place can lead to clinician reporting bias. There is no doubt from the many studies undertaken to

date that difficult embryo transfers occur less frequently than easy ones, but finding an exact figure is almost impossible without systematically reviewing the literature. Examining the literature, it would suggest that 'difficult' ET prevalence lies somewhere between 0.5-40 % of all procedures. This is clearly an inaccurate estimate that varies considerably according to how you define and grade embryo transfer difficulty. It will be important in this thesis to carefully examine the literature and both define and report a more accurate interpretation of prevalence.

1.3.3 Predictors

How a clinician sets about predicting a difficult ET seems to be a key question that remains unanswered. It is well accepted that when an embryo transfer is not easy there is likely to be a detrimental impact on pregnancy rates. Therefore, it is crucial to IVF success rates to have a reliable method to predict a difficult embryo transfer and avoid it happening. Unsurprisingly one of the most important factors to consider is the subjective opinion of the clinician undertaking the ET of the degree of difficulty expected (Lass et al., 1999).

There are many factors that a clinician may consider as relevant when predicting difficult ET. Each clinician undoubtedly places different amounts of importance on each factor and this was shown in a study of Austrian clinicians (Kovacs, 1999). Kovacs et al. (1999) designed a matrix of 12 factors that they felt may impact on the ET procedure potentially affecting IVF success. This study is somewhat dated now, but the principle was to try to work out which of these independent factors the

clinicians consider to be important. The presence of blood on the ET catheter, touching the fundus of the uterus and ET catheter choice were all high on the list of concerns.

Mains et al. (2010) more recently have summarised the many factors that have been shown to influence the embryo transfer technique. Specifically, they state that the main reasons for a difficult transfer are cervical stenosis or a large degree of anteversion/retroversion or anteflexion/retroflexion of the uterus (Mains and Van Voorhis, 2010). Whilst these factors have been shown in studies to be surrogate markers of 'difficult' ET it is hard to be certain how important they are.

Larue et al. (2017) published a study during this research that attempts to define and validate the anatomical causes of difficult ET procedures. This was an observational study in 306 IVF patients comparing the frequency of anatomical features in the cervix and uterus in patients with 'easy' and 'difficulty' transfers. This study revealed that abnormal crypts in the cervical canal and a tortuous cervical canal were the two most common anomalies that relate to 'difficult' ET (Larue et al., 2017). Whilst the problem of predicting when a patient will have a difficult ET is beyond the scope of this body of work, the issue has still to be resolved and will be considered alongside my findings.

1.3.4 Techniques to overcome

Several of the techniques that have already been reviewed here such as; catheter choice, mock ET, ultrasound-guidance, bladder filling, are all used to minimise the difficulty of ET. However, there have been other suggestions that are far more invasive that have been tested with the aim of overcoming difficult embryo transfer.

Mahajan et al. (2013) concluded that the use of 'Versapoint' electrocautery to refashion the cervical canal in extremely difficult ET patients did improve the ease of embryo transfer (Mahajan and Gupta, 2011). Versapoint electro-cautery to refashion the cervix is not frequently reported and a step further than hysteroscopy and cervical dilatation, and must be a last resort. This technique may initially reduce ET difficulty but may have damaging effects for future ET procedures with scarring and adhesions potentially forming in the cervical canal. Further work is required to clarify the important predictive factors and how to avoid difficult ET.

1.3.5 Grading ET difficulty

The literature shows very little consistency in its approach to grading ET difficulty. Whilst a clinician will certainly have an inherent feeling about when an ET procedure has 'not been easy', this is subjective. There are studies in the literature that have taken a dichotomous approach to grading difficulty describing just 'easy' and 'difficult' transfer procedures (Awonuga et al., 1998b, Mansour et al., 1990, Sallam et al., 2002). Other studies have used multiple levels to grade 'difficult' and address

the fact that this subjective measurement is on a continuous spectrum. The lack of consensus is evident though with studies describing anything from two to six levels of ‘difficulty’, and frequently using different criteria at each level.

A recent study published by Kava-Braverman et al (2017) has reassuringly shown in a study of more than 7,714 embryo transfer procedures that clinical pregnancy rates decrease progressively with the use of additional manoeuvres. Therefore, performing stepwise manoeuvres systematically with increasing levels of difficulty may be a reliable way to grade ET (Kava-Braverman et al., 2017).

In terms of finding a universally applicable, reliable and accurate system to classify the difficulty of all ET procedures there is much work to be done. The current definitions and grading scales are not fit for purpose and questions remain about what exactly is meant by a ‘difficult’ ET.

1.4 SUMMARY & HYPOTHESES

The above literature review has highlighted the fact that embryo transfer is an important step in an IVF cycle and can affect pregnancy outcomes. In summary, there is no clear definition of what is mean by a 'difficult' embryo transfer and no evidence based system or grading scale in place to allow clinicians to compare ET 'difficulty' easily. There are several different ET catheters for replacing embryos in the uterus and this coupled with the fact that clinicians frequently adopt their own personal technique makes it even more challenging to address. A myriad of different factors has been associated with ET 'difficulty' as has been described here, but there is still no consensus on how best to define it. Multiple studies over more than thirty years have resulted in what appears to be heterogeneous data sets that provide inconsistent and conflicting opinions. As a result, there is still no deep understanding of why the ET technique affects an embryo's implantation potential.

There is a need to search for a universally applicable, reliable and clinically useful system for measuring 'difficult' embryo transfer procedures, if research is to advance in this important area and discover what is happening to a single embryo's potential to implant. The overall aim of my research is to investigate whether an interchangeable ET 'difficulty' grading system can be found to improve clinician reporting and advance our understanding of how embryo transfer affects the implanting embryo. To test my hypotheses, the following specific objectives will be addressed:

1.4.1 Aims & hypotheses

1. Define what is meant by ‘difficult’ ET and calculate its prevalence (Chapter 2).
2. Develop a reliable alternative system to both describe and measure ‘difficult’ ET that is different to the current definitions and grading scales (Chapter 3).
3. Quantify the impact that a prolonged embryo transfer time has on embryos during ‘difficult’ embryo transfer cycles (Chapter 4).
4. Examine how prolonged exposure time impacts on the embryo by studying this effect in a mouse model and identify the relationship this has with its potential to implant (Chapter 5).
5. Determine the reliability and accuracy of a novel ET ‘difficulty’ grading scale designed for widespread use in a clinical environment (Chapter 6).

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Chapter 2

Defining Difficult Embryo Transfer and Prevalence

2.1 Introduction

2.1.1 What is 'difficult' embryo transfer and how common is it?

'Difficult' ET is described widely throughout the literature and it is a phenomenon that is well accepted to affect IVF outcomes. The prevalence of difficult embryo transfer is unclear as several different systems have been used to both grade and define 'difficulty'. A systematic review of the literature by Phillips et al. in 2013 looked at how difficult embryo transfers impact on clinical pregnancy rates. It neatly concluded that the main issue limiting progress in this area is that there are so many different definitions used to describe difficulty (Phillips et al., 2013b). Even though this important final step of the IVF treatment cycle impacts on pregnancy outcomes there is no uniform definition or scale against which clinicians can measure or record difficulty.

Despite more than thirty years of research looking at embryo transfer 'difficulty' there is no consensus on the best way to define and measure it. Reporting of ET 'difficulty' relies entirely on the clinician's subjective interpretation of a clinical procedure and unsurprisingly as a result there appears to be significant heterogeneity both in clinical techniques and in the reporting of 'difficulty'. As with any subjective measure the reliability and validity of that measurement relies on clearly defined boundaries both to inform and improve the consistency and rigour of the measurement taken.

There have been several surrogate markers that have been associated with and used to measure ET 'difficulty' in the past such as: blood on the catheter, cervical traction, the need for a stiff catheter, cervical dilatation, catheter resistance and the need for additional manoeuvres. This last factor was recently reported by Kava-Braverman et al. (2017) in a large retrospective observational study and showed that the progressive addition of manoeuvres during ET impacts on implantation significantly. Previously studies appear to have ignored the much lesser ET catheter manoeuvres and adjustments made by the clinician and have focussed on the key factors described here, such as blood on the catheter or use of a tenaculum. It is of interest to look at these subtler factors more closely to see if they also impact on the implanting embryo.

The challenge facing clinicians and researchers studying the effects of ET in IVF cycles is that different clinics use different ET catheters and techniques to replace embryos. Adopting a 'one-size-fits-all' approach to grading the difficulty of a procedure is not easy in an environment where practice is so varied. This magnifies the challenge when trying to understand exactly what is meant by 'difficult' ET and calculating prevalence. Future research should consider the entire spectrum of manoeuvres and the subtler markers that occur during ET that are consistently measurable and may affect an implanting embryo. Only then with a proper consensus can a system be designed that may be applied universally in all clinics. This chapter sets out to look systematically at the existing literature and examine the terminology that has been used over the years to define ET difficulty and help calculate the prevalence of this condition.

2.1.2 Materials & Methods

2.1.2.1 Eligibility criteria

Inclusion criteria were any study where 'difficult' ET was defined and interpreted in the context of human subjects undergoing IVF and prevalence determinable as either a primary or secondary outcome measure. All conference abstracts were considered. Letters to the editor or review articles were excluded from analysis. There was no limitation on publication date or language used. Suitability was determined by screening the titles and abstracts initially, but if this did not answer the question then the full article was obtained. Reference lists from included articles were screened for any articles that may have been missed in the initial search.

2.1.2.2 Information sources

The following databases were searched electronically on 12th June 2017: Cochrane Central Register of Controlled trials (CENTRAL), Medical Literature Analysis and Retrieval System Online (MEDLINE) and Embase spanning the years 1946 to 2017.

2.1.2.3 Search terms

The following search terms were used, adjusting for each database as necessary: embryo transfer difficulty*, difficult embryo transfer*, difficult transfer*, difficulty of embryo transfer, easy embryo transfer*, ease of transfer*, easy transfer*, embryo

transfer blood*, embryo transfer tenaculum*. MESH terms were expanded to gain the maximum number of options.

2.1.2.4 Study selection

Two independent reviewers screened the retrieved titles and abstracts selecting and excluding those that clearly did not meet the eligibility criteria. Disagreements were resolved with consensus from a third party. The full articles were then retrieved for all potentially relevant studies (n = 130), and then examined by two reviewers again resolving any disagreements over inclusion or exclusion by using a third expert. Studies were then finally included for further analysis if they related to human subjects and met all eligibility criteria. All primary articles included in the systematic review (n = 30) were assessed for the following: study design; appropriate sampling and adequate description of population characteristics (Figure 2.1.2-1)

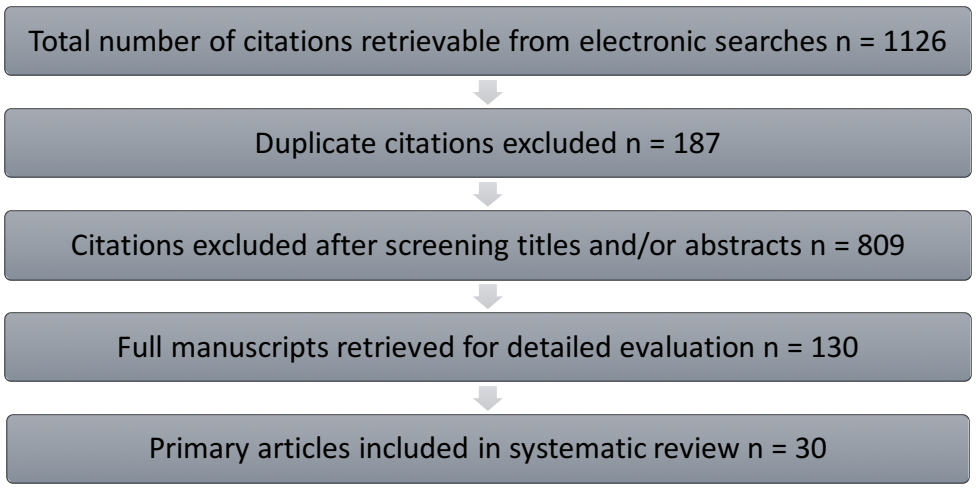


Figure 2.1.2-1 The study selection process for the systematic review on the prevalence of difficult embryo transfer

2.1.2.5 Data collection process

The data collection was performed independently by two reviewers. Data extracted included: study design, size, location, intervention, inclusion and exclusion criteria, ET details (number of embryos transferred, type of catheter used, ultrasound guidance), the number of difficulty levels reported, the definitions for each level of difficulty and the proportions of embryo transfers categorised at that level. The primary reproductive outcomes for each included study were also collated during data collection and extraction where possible.

Investigators studying ET regularly use different levels of 'difficulty' to describe the ease of transferring an embryo back into the uterus. Studies differed considerably in how they defined these difficulty levels and there was a definite distinction between studies that had precisely defined 'difficulty' levels and those that were less clear, relying on a clinician's subjective interpretation of the actual difficulty of the ET procedure. Studies that stratified difficulty 'optimally' presented a clear definition with no room for subjectivity and so were regarded as more reliable and less susceptible to clinician interpretation bias. 'Sub-optimal' studies relied heavily on the clinician's subjective interpretation of the ET procedure and usually defined 'difficult' ET less clearly with the use of terminology such as; 'requiring a strong push', 'increased force', 'increased time' and 'requiring manipulations (Table 2.1.3.5-1). Data was collated but also considered separately in these two groups; (1) 'optimal' and (2) 'suboptimal', when assessing the prevalence of difficult ET.

Table 2.1.2.5-1 The 'optimal' and 'sub-optimal' definitions used to describe difficult ET in all included studies and the number of criteria required to meet the 'difficulty' level

'Optimal' definitions	Criteria
Unable to pass catheter immediately needing cervical dilatation	2
Requires a tenaculum or metal catheter	1-2
Advance outer sheath or use stiffer catheter, + use tenaculum	2-3
Unable to pass catheter immediately + catheter moulding or tenaculum used	2-3
Hard catheter required or tenaculum used	1-2
Hysterometer needed	1
Multiple attempts or cervical dilatation	1-2
Resistance requiring tenaculum, retained embryos or change of catheter	1-3
Tomcat catheter used + either tenaculum and/or dilators used	2-3
Presence of blood/mucus, and/or embryos reloaded, and/or catheter change	1-4
Tenaculum used	1
Soft Wallace failed to advance and firm stylet required	1
Needed an os-finder and/or tenaculum in addition to obturator to pass	2-3
Requiring a firm stylet to pass	1
Use of tenaculum, and/or blood on sheath/catheter, and/or uterine sound	1-3
Instrumentation with either tenaculum/hysterometer	1-2
'Sub-optimal' definitions	**Criteria**
Long period of manipulation and force needed and bleeding usually seen	3
Catheter requires a 'strong' push or 'manipulations' to pass	1-2
Requiring uterine manipulations, increased force, tenaculum, traumatic	4
Tomcat catheter needed, dilatation, bleeding, extreme difficulty	1-4
Requiring uterine manipulations or increased force or cervical grasping and/or accompanied by trauma	1-4
Tomcat catheter needing cervical manipulation (requiring a tenaculum to straighten cervical canal and/or significant sheath threading)	2
Either a change or catheter, and/or negotiation of cervix, and/or use of volsellum, and/or retained embryos, trans myometrial transfer	1-5
Greater resistance was met, the procedure was time consuming, need to change to harder catheter, uterine sounding or cervical dilatation, or blood	1-5
Requiring uterine manipulations, increased force, tenaculum, traumatic	4
Taking considerable time to enter uterus, +/- tenaculum or change catheter	2-3
Difficulty inserting catheter due to UCA, cervical stenosis or time > 5 mins	1-3
Use of a cervical dilatator or reconversion of the intended technique	1-3
Manipulation or sounding of uterus required	1-2
Additional instrumentation required or change catheter	1-2
Use of force and/or additional instruments and/or manipulation	1-3
Time increased or additional techniques such as stylet or tenaculum needed	1-3

Data was extracted from studies and analysed collectively but also in sub-groups according to the terminology used to define 'difficult'. Studies that had defined 'easy' ET were also analysed for prevalence and levels of heterogeneity.

2.1.2.6 Statistical analysis

Statistical analysis was performed using Stata 14.0 statistical software (Stata corp, Tx, USA). Meta-analyses were performed to establish the prevalence of 'difficult ET' and this was calculated for all included studies but also separately for key sub-domains of 'difficulty' (e.g. blood on the catheter, use of a tenaculum) distinguishing for the data sets extracted from 'optimal' and 'suboptimal' studies. For meta-analyses, proportions were pooled weighting each study by the inverse of its variance, and the summary estimates displayed. A DerSimonian-Laird random-effects model was used for analysis and heterogeneity assessed using Cochrane's Q and I_2 statistic.

2.1.3 Results

The literature search revealed 1126 citations; of which 187 duplicates were excluded (Figure 2.1-1). Careful assessment of title and abstract resulted in the exclusion of a further 809 citations that did not fulfil the selection criteria. Full manuscripts were obtained for the remaining 130 citations and following further scrutiny 30 articles were identified as relevant to the systematic review. The thirty studies in total included 36,547 women undergoing ET.

Studies were analysed in sub-groups based on the number of difficulty levels used by the clinician to grade ET 'difficulty'. 15 studies defined ET difficulty on two levels, giving the clinician just two options to choose between; 'easy' or 'difficult'. 10 studies stratified difficulty on 'three' levels, giving the clinician three options to choose from when grading the ET procedure, so in these studies when a procedure wasn't 'easy' the increasing difficulty was defined over two further levels. In the remaining 5 studies ET difficulty was defined across four levels leaving the clinician three more levels above 'easy' to consider when rating ET 'difficulty'. Pooled prevalence rates for this sub group analysis is summarised in Table 2.1.3.5-1. and raw data in Appendix 2.

2.1.3.1 'Easy' Transfers

To fully appreciate the problems that exist in the classification of 'difficult' ET it is useful to first look at the opposite end of this spectrum. 'Easy' ET was reported and defined in all 30 studies included in this review. Definitions tended to be very similar and used terms such as; 'no force required', 'smooth passage', 'no additional instrumentation, 'without effort', 'no delay'. The overall pooled prevalence rate for 'easy' ET in the included studies was 82.7% (95% CI 79.5-85.9%). The degree of study heterogeneity was low (I_2 = 17.74) for study estimates of the prevalence of 'easy' ET

2.1.3.2 'Non-easy' transfers

Whilst most studies defined an 'easy' ET procedure consistently, as evidenced by the lower levels of heterogeneity across the included studies, there is little doubt that the rest of the ET procedures, were therefore 'non-easy' by default. Significant heterogeneity (I_2 = 78.56) exists in this collective pooling of all 'non-easy' ET procedures with the prevalence reaching 15.5% overall (95% CI 12.8-18.2), demonstrating that studies used very different grading structures to record the 'non-easy' transfers with little consistency in the definitions at each level of difficulty.

2.1.3.3 'Two-levels' of difficulty

Studies defining ET 'difficulty' over just two levels clearly defined 'easy' ET and then in most cases grouped several descriptive criteria together to define the second level of ET 'difficulty' (Table 2.1.2.5-1). The pooled prevalence rate for 'difficult' ET in this sub-group was 14.3% (95% CI 10.5 – 18.0%) and the degree of heterogeneity was high (I_2 = 78.8%) suggesting high variation across study estimates. The pooled prevalence in the 'optimal' studies that had used a much clearer definition of 'difficult' was 16.9% (95% CI 11.3 -22.5%) and heterogeneity remained high (I_2 = 78.3%) (Wisanto et al., 1989, Awonuga et al., 1998b, Meriano et al., 2000, Sallam et al., 2003, Ciray et al., 2007, Tiboni et al., 2012a, Ghanem et al., 2015, Kava-Braverman et al., 2017). The second level of difficulty was defined 'sub-optimally' in 7 out of 15 studies, increasing the risk of bias due to clinician interpretation of these subjective terms (Mansour et al., 1990, Sallam et al., 2002, Spandorfer et al., 2003,

Eskandar et al., 2007, Bodri et al., 2011, Singh et al., 2012, Ghaffari et al., 2013). The seven 'sub-optimal' studies that had defined difficulty less concisely were more at risk of clinician interpretation bias and the pooled prevalence was 11.5% (95% CI 6.8-16.2) with significant heterogeneity (I_2 = 76.3%).

2.1.3.4 'Three-levels' of difficulty

Ten studies had adopted three-levels of difficulty and had clearly defined 'easy' ET in all cases, and then diluted the 'frequently used' terms to describe higher levels of difficulty across two further levels (Figure 2). Unsurprisingly the pooled prevalence, when three-levels of difficulty were interpreted by clinicians, resulted in a lower pooled prevalence of 3.7% (95% CI 1.7-5.5%). Seven studies were assessed as having 'optimally' described 'difficulty' levels (Kan et al., 1999, Garcia-Velasco et al., 2002, Allahbadia et al., 2010a, Flisser et al., 2006, Listijono et al., 2013, Mirkin et al., 2003, Yilmaz et al., 2013), while 3 studies had 'sub-optimally' defined levels of difficulty (McIlveen et al., 2005, Plowden et al., 2017, Tomas et al., 2002). The pooled prevalence was found to be 3.8% (95% CI1.3-6.2, I_2 = 77.3%) and 3.6% (95% CI 0.6-6.5, I_2 = 47.4%) respectively for both the 'optimally' and 'sub-optimally' designed studies, demonstrating marked heterogeneity despite the supposedly improved quality of the 'optimally' described grading systems.

2.1.3.5 'Four-levels' of difficulty

Five studies that had defined four-levels of difficulty had in all cases defined 'easy' in a consistently interpretable way, leaving very little room for uncertainty or subjective interpretation bias. However, over three further difficulty levels a selection of different terms were used independently or collectively to define increasing levels of 'difficulty' (Table 2.1.2.5-1). Heterogeneity remained predictively high across these studies and the pooled prevalence overall in this group was 6.8% (95% CI 3.1-10.4, I_2 = 63.9). Two 'optimal' studies showing a lower prevalence 3.7% (95% CI 1.1-6.4, I_2 = 43.4) (Matorras et al., 2004, Spitzer et al., 2012) and three 'sub-optimal' studies predicted a much higher pooled prevalence of 9.8% (95% CI 3.9-15.8, I_2 = 66.3)(Leeton et al., 1982, Noyes et al., 1999, Tur-Kaspa et al., 1998).

Table 2.1.3.5-1 Pooled prevalence of sub-groups according to number of allocated ET difficulty levels and whether ET grading criteria was 'optimal' or 'sub-optimal'

Group	No. studies	No. Patients	Pooled Prevalence	95% CI	I_2
ALL 'Easy' ET	30	36,547	82.7	79.5-85.9	17.7
ALL 'Non-easy' ET	30	36,547	15.5	12.8-18.2	78.5
ALL 'Difficult' ET	30	36,547	9.6	7.4-11.8	86.2
• *optimal*	17	23649	9.2	6.3-12.2	88.5
• *suboptimal*	13	12896	9.0	6.0-12.0	77.7
'Two levels' ET difficulty	15	16606	14.3	10.5-18.0	78.8
• *optimal*	8	11762	16.9	11.3-22.5	78.3
• *suboptimal*	7	4844	11.5	6.8-16.2	76.3
'Three levels' ET difficulty	10	16196	3.7	1.7-5.5	71.6
• *optimal*	7	10317	3.8	1.3-6.2	77.3
• *suboptimal*	3	5879	3.6	0.6-6.5	47.4
'Four levels' ET difficulty	5	3745	6.8	3.1-10.4	63.9
• *optimal*	2	1570	3.7	1.1-6.4	43.4
• *suboptimal*	3	2175	9.8	3.9-15.8	66.3

Table 2.1.3.5-2 Primary studies included in the systematic review of the prevalence of difficult embryo transfer

Study ID	Study location type, size	Intervention	Inclusion / exclusion	No. difficulty levels	Difficulty levels	Level 1 (Easy)	Level 2	Level 3	Level 4	Optimal / Suboptimal
Leeton et al. 1982	UK based, randomised trial, n=204	catheter type	Fresh IVF cycles	4	Easy, suspect, difficult, failed	delivery of embryos at first attempt with little or no mucus	some difficulty passing internal os, uncertainty of catheter tip position +/- blood or mucus	long period of manipulation to pass catheter through cervix, force often needed and bleeding usually seen	unable to access cavity	Sub-optimal
Wisanto et al 1989	Belgium, prospective randomised, n =400	catheter type	Fresh IVF cycles	2	Excellent or fair, difficult	catheter passed easily	unable to pass catheter immediately needing cervical dilatation.			Optimal
Mansour et al. 1990	Egypt, case controlled, n=335	Mock transfer	Fresh IVF cycles	2	Easy, difficult	catheter passes easily through the cervix without resistance	catheter requires a strong push or manipulations to pass			Sub-optimal
Awonuga et al 1998	UK, n=1204		Fresh IVF cycles	2	Easy, difficult	straightforward	requiring tenaculum or metal catheter			Optimal
Tur-kaspa et al. 1998	Israel, n= 854, prospective cohort study			4	Easy, difficult, cervical dilatation, multiple attempts	smooth, unforced	requiring uterine manipulation, or increased force, or tenaculum, and/or traumatic	cervical dilatation required	several attempts required	Sub-optimal

Study ID	Study location type, size	Intervention	Inclusion / exclusion	No. difficulty levels	Difficulty levels	Level 1 (Easy)	Level 2	Level 3	Level 4	Optimal / Suboptimal
Kan et al. 1999	UK, n=187, prospective control study	US v clinical touch	Fresh IVF cycles, excluded = >42, ET under GA, > 3 cycles	3	Very easy, easy, difficult	transfer catheter went straight through the cervix	advance the outer sheath or a stiffer catheter	advance the outer sheath or a stiffer catheter + required a tenaculum		Optimal
Noyes et al. 1999	USA, retrospective observational , n=1117	Surgical correction of cervical stenosis	IVF fresh <40yrs	4	Easy, moderate, difficult, very difficult	no effort	Wallace + tenaculum, advance outer, use of TOM cat some blood (speck)	TOMcat catheter to replace embryos, with blood + tenaculum	TOMcat cath, dilatation, bleeding, extreme difficulty	Sub-optimal
Meriano et al. 2000	Canada, prospective randomised, n=66	Catheter type	<38 yrs. IVF/ICSI cycles	2	Easy, difficult	not difficult	catheter not introduced immediately and manipulations, e.g. changing the curvature of the catheter or use of a tenaculum			Optimal
Garcia-velasco et al. 2002	ESP, prospective randomised study, n=374	US guided v clinical touch	IVF donor cycles	3	easy, difficult, very difficult	soft catheter passed smoothly through the cervix	an outer teflon sheath required	rigid catheter required or tenaculum		Optimal
Mattoras et al. 2002	ESP, quasi-RCT, n =260	US guided v clinical touch	Fresh IVF/ICSI,	4	Easy, intermediate, difficult, very difficult	soft catheter only needed	catheter manipulation, tenaculum	hysterometer needed	multiple catheter changes, cervical dilatation, local anaesthetic	Optimal

Study ID	Study location type, size	Intervention	Inclusion / exclusion	No. difficulty levels	Difficulty levels	Level 1 (Easy)	Level 2	Level 3	Level 4	Optimal / Suboptimal
Sallam et al. 2002	EGYPT, prospective case-controlled, n=640	US + mould catheter to UCA	Fresh IVF/ICSI,	2	cervical resistance, tenaculum, retained embryos, change catheter,	No difficulties recorded	change or catheter, and/or negotiation of cervix, and/or use of volsellum, and/or retained embryos, trans myometrial transfer			Sub-optimal
Tomas et al. 2002	POR, 3-centre retrospective observational analysis of ET, n=4807		IVF/ICSI cycles	3	Easy, intermediate, difficult	smoothly, without the use of any instrumentation, the catheter was clean of blood and there was no need to change the catheter	the primary catheter met some resistance, leading to the use of cervical forceps and/or the outer sheath catheter, after which the transfer was smooth and there was no blood contamination	greater resistance was met, the procedure was time consuming, there was a need to change to a harder catheter, uterine sounding or cervical dilatation was carried out or there was blood in any part of the catheter		Sub-optimal
Mirkin et al. 2003	USA, retrospective observational , n=823	US guided v clinical touch		3	very easy, easy, difficult	no difficulty at all	catheter manipulation needed, advance outer co-axial sheath, tenaculum	multiple attempts or cervical dilatation		Optimal
Sallam et al. 2003	EGYPT, cohort study, n=784	Catheter choice, blood	IVF/ICSI cycles	2	cervical resistance, tenaculum, retained embryos, change catheter,	catheter entered the uterine cavity smoothly on the first attempt	need to: hold the cervix with a volsellum, change the catheter, or have two consecutive attempts (retained embryos)			Optimal

Study ID	Study location type, size	Intervention	Inclusion / exclusion	No. difficulty levels	Difficulty levels	Level 1 (Easy)	Level 2	Level 3	Level 4	Optimal / Suboptimal
Spandorfer et al. 2003	USA, case-controlled, n=2263	Mock followed by ET and catheter change	IVF fresh cycles	2	Easy, difficult	catheter passes easily through the cervix without resistance	requiring uterine manipulation, increased force, tenaculum, traumatic			Sub-optimal
McIlveen et al 2005	UK, prospective RCT, n = 150	catheter choice	Fresh IVF	3	Easy, moderate, difficult	no difficulty or delay into the cavity	some time may have been taken to negotiate the cervical canal or an obturator may have been needed	taking considerable time to enter the uterus, requiring a tenaculum, or requiring a change of catheter		Sub-optimal
Flisser et al. 2006	USA, retrospective comparison study, n = 249	US guided v clinical touch	Fresh IVF cycles	3	Easy, moderate, difficult	embryos placed without effort	Wallace with tenaculum or TomCat without difficulty	TomCat (firm) cath. to replace embryos, dilators or tenaculum		Optimal
Ciray et al. 2007	Turkey,	Time of transfer	<38 yrs. IVF/ICSI cycles, no PGD, no recurrent failed cycles (>2)	2	Easy, difficult	No blood/mucus, no reloading embryos, no catheter change required	Blood, mucus, reloading embryos or catheter change			Optimal
Eskander et al. 2007	Saudi, prospective controlled trial, n = 286	Aspiration of cervical mucus	Fresh ET cycles	2	Easy, difficult	not described	difficulty placing catheter in uterine cavity due to UCA, cervical stenosis OR if ET took > 5mins			Sub-optimal

Study ID	Study location type, size	Intervention	Inclusion / exclusion	No. difficulty levels	Difficulty levels	Level 1 (Easy)	Level 2	Level 3	Level 4	Optimal / Suboptimal
Allahbadia et al. 2010	Israel, prospective randomised study, n = 175	Random use of either SureVew or Wallace cath	Donor egg cycles	3	very easy, easy, difficult	catheter passes smoothly	advance stiff co-axial	tenaculum used		Optimal
Bodri et al. 2011	Spain, RCT n = 330	TV v TA US guidance	Donor egg cycles	3	Without any difficulty, moderate difficulty, highly difficult	smooth introduction of the catheter	longer, more difficult, or repeated introduction of the catheter	use of a cervical dilatator or reconversion of the intended technique		Sub-optimal
Singh et al. 2012	India, retrospective study, n = 342	Easy v Difficult	Fresh IVF/ICSI	2	Easy, difficult	not described	additional instrumentation required or firmer catheter or change of catheter			Sub-optimal
Spitzer et al. 2012	Austria, retrospective, n = 1055	ET difficulty	IVF/ICSI/IMSI	4	ET1, ET2, ET3, ET4	atraumatic using just soft inner	advancing outer sheath first and loading soft inner after	using firm stylet	using a tenaculum	Optimal

Study ID	Study location type, size	Intervention	Inclusion / exclusion	No. difficulty levels	Difficulty levels	Level 1 (Easy)	Level 2	Level 3	Level 4	Optimal / Suboptimal
Tiboni et al. 2012	Italy, retrospective case controlled, n = 553	Malleable stylet versus Soft approach	Patients <37 yrs. with TET	2	With stylet, Without stylet	Wallace without need for firm stylet	required firm stylet			Optimal
Ghaffari et al. 2013	Iran, prospective study, n = 706	Easy v Difficult	Included: < 41yrs, FSH <15, >1 good quality D2/3 embryo. Excluded: any abnormal pathology uterus/cervix, D5 ET or FER, egg donation or surrogacy	2	Easy, difficult	Without the use of force or instrumentation	use of force for catheter placement and/or the use of additional instruments and/or manipulation			Sub-optimal
Listijono et al. 2013	Australia, population based study, n = 6484	ET difficulty	Fresh and Frozen IVF/ICSI cycles	3	easy, medium, difficult	smooth procedure without obturator	obturator required	obturator, os finder, tenaculum		Optimal
Yilmaz et al. 2013	Turkey, retrospective, n = 313	Dual loading technique	Fresh SET ICSI cycles, excluded: tenaculum use	3	1,2,3	easy transfer with a direct soft catheter	afterload with external guidance	required a firm stylet		Optimal

Study ID	Study location type, size	Intervention	Inclusion / exclusion	No. difficulty levels	Difficulty levels	Level 1 (Easy)	Level 2	Level 3	Level 4	Optimal / Suboptimal
Ghannem et al. 2015	Egypt, retrospective cohort, n = 744	Surrogate markers of difficulty	Included <38yrs, Fresh ICSI, first cycle, >2 HIGH quality embryos transferred	2	Easy, difficult		cervical traction, blood on outer sheath/ET catheter, or uterine sound			Optimal
Kava-Braverman et al. 2017	USA< retrospective analysis, n= 7714			2	Easy, difficult	direct ET or outer sheath advanced or Wallace malleable	tenaculum or hysterometer			Optimal

**Full manuscripts were screened by Dr E. Coats and Dr S. Asif*

2.1.4 Discussion

This systematic review assesses the prevalence of ET 'difficulty' in a population of patients undergoing assisted reproductive treatment with IVF or ICSI. It is the first review in the literature to evaluate pooled prevalence in this way. It also considers studies with 'optimal' and 'sub-optimal' definitions of 'difficult' separately, as the subjectivity of this measurement is the main issue facing clinicians in the clinic. Clinician reporting bias was anticipated to be more frequent in these 'sub-optimal' studies where ET 'difficulty' levels had been poorly defined. It was therefore important to differentiate between studies in this way to improve the accuracy of the results.

2.1.4.1 Principal findings

This review has shown that the prevalence of 'easy' ET was 82.7% and when all 'non-easy' ET procedures were considered collectively the prevalence was 15.5%. Combining all the 'non-easy' procedures in this way is a simple way to gauge the total proportion of procedures that were more 'difficult' to complete. However, this definition of 'difficult' encompasses all ET procedures of mixed levels of difficulty. Whilst it is a useful collective term it does not differentiate for the lesser difficulties that occur during an ET procedure. This pooled prevalence gives us a general idea of how many procedures were not straightforward requiring additional manoeuvres to bypass resistance. It can be concluded that a 'difficult' ET involving additional manoeuvres to overcome catheter resistance has a prevalence of 15.5% but this

measurement is associated with considerable heterogeneity. The important question, which this thesis attempts to address, is what is the best way to define and grade the varying levels of 'difficult' ET.

2.1.4.2 Limitations of the review

This review looks retrospectively at thirty studies, with significant heterogeneity of the techniques used to transfer embryos and how they define levels of 'difficult' ET. Analysis is therefore limited by this and the fact that 'difficulty' is reported in descriptive terms in studies and as a subjective variable this is susceptible to interpretation and reporting bias.

2.1.4.3 Strengths of the review

This review used an extensive electronic search of all peer reviewed studies through Cochrane Central Register of Controlled trials (CENTRAL), Medical Literature Analysis and Retrieval System Online (MEDLINE) and Embase, as well as including all conference abstracts. Attempts were made to tackle the significant levels of study heterogeneity by dividing included studies into 'optimal' and 'sub-optimal' sub-categories. One of the many problems in the literature is the many different systems used to classify different levels of difficulty and this review addressed this by grouping studies by the number of tiers of difficulty that had been used.

2.1.4.4 What is 'optimal'

No two studies used the exact same system to describe ET 'difficulty'. To address the high levels of study heterogeneity this review looked at the many different definitions used to define 'difficult' ET and selected which ones were 'optimal' based on the how susceptible they were to prejudice by the clinician. Some studies had used clear criteria to define difficulty levels, which made it harder for clinicians to misinterpret and incorrectly allocate a difficulty level for an ET procedure.

The system used to select 'optimal' studies was determined by how reliably clinicians would likely report ET 'difficulty', based on the criteria outlined in the study's grading system. However, this system does not consider the multiple terms used by different investigators to define each level of difficulty, which is certainly another source of heterogeneity within groups. In general, the studies that defined difficulty 'optimally' tended to have fewer criteria in their definition at each level (Table 2.1.2.5-1) and used more explicit terms such as; a tenaculum was applied, required a firm stylet to pass, a hysterometer was needed.

Although 'optimal' studies used much clearer definitions, significant heterogeneity remains due to the inconsistent allocation of descriptive terms at each level of difficulty. Ciray et al (2007) for example used an 'two-level' system and described difficulty 'optimally' when between 1-4 criteria were met and the "presence of blood/mucus, and/or embryos reloaded, and/or a catheter change" was noted (Ciray et al., 2007). Ghannem et al. (2015) also used a 'two-level' system but optimally

defined difficult ET as occurring when between 1-3 criteria had been met and the "use of a tenaculum, and/or blood on sheath/catheter, and/or uterine sound" was noted. These two examples are representative of many of the included studies in this review as there was no real pattern identified as to how investigators had integrated different criteria into different difficulty levels.

In most cases, it was not possible to calculate the prevalence of these individual surrogate markers easily. Ghannem et al. (2015) is an exception as it had broken 'difficult' down even further, indicating the proportion of transfers resulting in 'blood on the catheter', 'use of a uterine sound' or 'a change of catheter'.

2.1.4.5 Surrogate markers of difficulty

Surrogate markers are frequently used to describe the difficulties associated with overcoming resistance when performing ET. The advantage of using surrogate markers like; the use of a tenaculum, use of a hysterometer, use of cervical dilatation, use of a rigid catheter, or the presence of blood on the catheter is that they are absolute. Many studies in this review have these surrogate markers integrated into their various descriptive scales of difficulty Table 2.1.3.5-2, and although in wide usage across the literature they result in marked heterogeneity. Despite these markers of difficulty being quite well-defined there appears to be no consensus across the literature of how to describe 'difficult' ET.

The problem with absolute markers such as ‘the use of a tenaculum’ is that this description doesn’t give any insight into the process that led up to choosing that instrument in the first place. A tenaculum applied at the start of a procedure may for example have a much lesser effect than a tenaculum applied after 5 minutes of struggling to overcome resistance and replace an embryo. It is known that the use of a tenaculum is likely to cause myometrial contractions which may result in lower pregnancy outcomes (Lesny et al., 1998), but the impact of the much lesser manoeuvres such as ‘advancing a co-axial sheath’ over a soft catheter are not always considered.

Most ET ‘difficulty’ grading systems that this review has examined don’t use a graded stepwise approach to catheter movements and only look at the opposite ends of the spectrum such as ‘easy’ transfers and markers such as ‘the need for a tenaculum’ for example. Further work is needed to also consider the lesser manoeuvres to determine their importance for an implanting embryo. It is necessary to look at each of those different markers and where possible assess their impact on reproductive outcomes to understand their significance and role in a modern up-to-date ET difficulty grading system.

2.1.4.6 ‘Difficult’ vs ‘non-easy’ and reproductive outcomes

This systematic review has shown that the prevalence of ‘non-easy’ ET is about 15.5% but as stated earlier this is likely to include procedures of varying levels of difficulty. As such it is almost impossible to determine accurately the true prevalence

of ‘difficult’ ET due to the marked heterogeneity across studies using different grading systems and definitions. Examining just the ‘optimal’ studies in this review, ‘difficult’ ET is estimated to lie somewhere between 3.7% and 16.9%, depending on how you define and grade it. Unravelling which markers of difficulty are most important in this ‘non-easy’ ET group will only be achieved by considering the impact they have on reproductive outcomes.

2.1.4.7 A consensus definition

This review has illustrated marked heterogeneity with the systems and definitions used to describe ET ‘difficulty’. It is only by studying the literature in this way that it becomes evident that there is no clear consensus on how best to define ‘difficult’ ET. The pooled prevalence of ‘non-easy’ ET is the best estimate at this stage of the proportion of patients that need extra help to replace their embryos. It is the aim of this thesis to try and achieve an improved and more scientific approach to defining and grading ET ‘difficulty’. However, the most accurate definition that can be offered at this stage is based on this review and all ‘non-easy’ ET procedures:

A difficult embryo transfer is any procedure where the ET catheter will not pass easily requiring additional manoeuvres to replace the embryo.

This broad definition gives a clear starting point to evaluate which factors affect all ‘non-easy’ transfers and more importantly may affect the implanting embryo and success rates.

2.1.4.8 Grading 'difficult' ET in practice

The challenge facing clinicians looking to develop a universal system that accurately grades all types of ET 'difficulty' is that clinicians use different techniques, different ET catheters and have different thresholds for using counter traction with a tenaculum or a hysterometer. Ideally any future ET 'difficulty' grading system should remain as independent as possible from these subjective markers yet continue to directly record the procedure in detail. A few studies in this review have considered that the 'embryo transfer time' (ETT) is an important marker of how difficult the procedure has been. Matorras et al. (2004) and Ciray et al. (2007) have both studied this variable with conflicting results. There are strengths and weaknesses to both these studies that warrant further work, which this thesis hopes to address.

2.1.4.9 Implications for future research

There is a real need for further work to try and develop a robust system to grade the difficulty of ET procedures for both clinical and research benefit. This study has shown that prevalence may lie anywhere from 3.6% to 16.9% due to the heterogeneity of the current difficulty grading systems. A consensus definition must be agreed and a simple systematic stepwise grading system must be found quickly that can accurately measure difficulty, is widely applicable and relates directly to IVF outcomes.

Certainly, the advantage to using a continuous measure like ETT is that it is directly applicable to all ET procedures, and is independent of the ET catheter choice and technique, though there is not much known about the impact this has on embryo potential. Whilst it may be influenced by the clinicians' ET technique the ETT will be less susceptible to clinician interpretation bias as it is simply a numeric record of the interval time taken from loading an embryo to discharging it into the uterus.

2.1.5 Conclusion

This systematic review has shown that study heterogeneity is high and whilst the prevalence of 'non-easy' transfers is around 15.5% the prevalence of 'difficult' transfer probably lies somewhere between 3.7% - 16.9%, and is hugely influenced by how it is defined and graded. A better grading system is required first to help research advance the understanding of why this last crucial step of the IVF cycle is so important for the implanting embryo. Perhaps the ETT combined with a more stepwise grading system considering the spectrum of catheter manoeuvres and not just the surrogate markers will more accurately and reproducibly record ET difficulty.

Chapter 3

Grading Embryo Transfer Difficulty

3.1 TESTING A UNIVERSALLY APPLICABLE GRADING SYSTEM

3.1.1 Introduction

Embryo transfer remains an important step during *in vitro* fertilisation (IVF) treatment (Schoolcraft, 2016). The ET procedure requires a skilled practitioner to pass an ET catheter through the cervix to replace the embryo in the uterus. Exactly what constitutes a 'difficult' ET is not clearly defined in the literature but it is of interest as it can be unpredictable and is reported to reduce IVF success rates (Tomas et al., 2002, Listijono et al., 2013). Chapter 2 attempted to define the prevalence of 'difficult' ET but concluded that an improved grading system is needed.

Increased resistance, prolonged transfer times, blood on the catheter tip, and use of a tenaculum have all been used to define 'difficulty' and are associated with suboptimal IVF outcomes (Goudas et al., 1998, Garzo, 2006, Lesny et al., 1999). The significance of these factors in the context of difficult ET is not well understood though as studies frequently include the transfer of multiple embryos at the cleavage or blastocyst stage in both fresh and frozen cycles. The number and quality of embryos replaced at transfer in addition to ET 'difficulty' is well accepted to affect pregnancy rates (Roseboom et al., 1995, Balaban et al., 2000). Therefore, the data are heterogeneous and can be difficult to interpret.

Chapter 2 identified that the prevalence of 'difficulty' is unclear as there is no standardised definition or grading system to describe ET difficulty consistently and reproducibly between clinics. Whilst difficult ET is accepted to be undesirable for the pre-implantation embryo little is understood about why this may lead to lower success rates. Inter-observer variability in the use of language and the interpretation of how difficult a procedure has been means that 'standardising' a grading system to cater for all ET catheters on the market and all ET techniques is a challenge (Kava-Braverman et al., 2017).

The aim of this experiment was to determine whether a continuous Visual Analogue Scale (VAS) may be a more reliable tool to grade embryo transfer (ET) difficulty when assessing IVF outcomes. This is the first study to determine whether a VAS provides a more consistent grading system than conventional descriptive narrative to report the difficulty of an ET procedure.

3.1.2 Materials & Methods

3.1.2.1 Ethics

After examination of the University of Nottingham policy of 'Guidance for Schools on the Ethical Review of Research', formal research ethics committee approval was confirmed as not be necessary for this work. This was a retrospective observational study and the data collected was obtained from anonymised patient records and involved no change or intervention to standard treatment protocols.

3.1.2.2 Methods

In November 2014, a continuous VAS (Figure 3.1.2-1) was added to the documentation note and clinicians were asked to record the difficulty of ET using the VAS in addition to using standard narrative. The VAS consisted of a 10-cm horizontal line and the clinician was instructed to place a vertical mark at the point that they believed best represented the level of difficulty. The left of the line (0-cm) represented the easiest transfer while the right of the line (10-cm) represented the most difficult transfer. In addition to marking on the VAS how difficult the ET had been, clinicians were asked to continue to record the ET procedure on the data collection sheet with specific attention to the stepwise approach taken to replace the embryo. Data was collected over a 17-month period on a standard proforma which included the VAS. All patients included in the study group underwent IVF or ICSI treatment with a long GnRH agonist or GnRH antagonist protocol and had a fresh day-5 single embryo transfer.

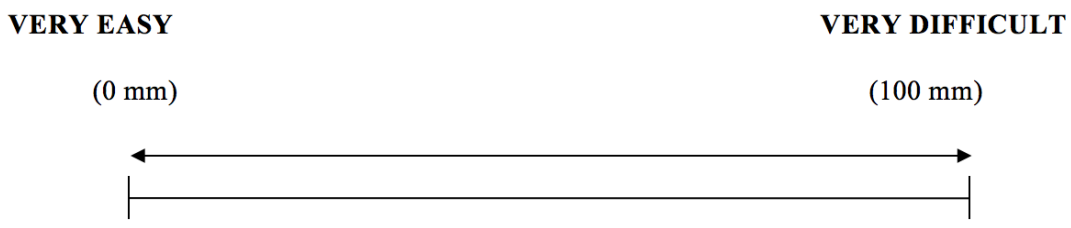


Figure 3.1.2-1 A Visual Analogue Scale (VAS) used to record embryo transfer difficulty by marking the scale based on subjective interpretation.

All clinicians in our unit underwent a mandatory six-month training period before undertaking embryo transfers and were taught a stepwise approach to embryo transfer. In all cases of single ET, the embryo was loaded into 20μl culture media between two air bubbles in an 18-centimetre soft embryo replacement catheter (Sure View® Wallace® Embryo Replacement Catheter CE118; Smiths Medical). ET was performed under ultrasound guidance and the first step was to pass the soft echogenic Wallace catheter loaded with the embryo into the cervix. If the catheter met resistance and would not pass, the next step was to advance the catheter's outer sheath beyond the tip of the inner catheter lumen, creating a firmer catheter to negotiate resistance. If there was continued high resistance, the clinician could then opt to use a firmer catheter (Malleable Stylet 1816ST; Smiths Medical) to bypass resistance in the cervix. Three different levels of increasing difficulty were identified (Table 3.1.2.2-1) based on these three logical steps taken by the clinicians. A fourth grouping emerged (Group M) where clinicians concerned about a difficult procedure adopted a two-stage after loading technique by inserting the firmer malleable stylet first followed by the soft catheter loaded with the embryo.

Table 3.1.2.2-1 Description of embryo transfer difficulty (A – Easy, B – Medium, C – Difficult, M- Malleable) based on the technique used to bypass resistance

A	Easy	No resistance to passing the catheter
B	Medium	Resistance overcome by advancing the outer sheath
C	Difficult	Malleable stylet used after failure to overcome resistance with outer sheath
M	Malleable	Electively malleable stylet is passed to internal os prior to embryo transfer

Each ET documentation record was scrutinised separately by two observers who interpreted the clinician's written notes. ET difficulty was categorised (groups A-C or group M) based on the conventional system of interpreting clinician narrative. When there was disagreement a third observer was asked to categorise the outcome based on the documented record and then the majority decision was final. The distance from the left border of the VAS was measured using a standard ruler to the nearest millimetre (0-100mm) and this correlated to a score between 0 and 100 for each ET. The VAS scores were ordered and re-categorised (Group 1-4) according to the 25^{th}, 50^{th} and 75^{th} percentiles to create four incremental groupings for further analysis.

Patient characteristics (age, BMI, ethnicity, smoking, and cycle number) and clinician key performance indicator (KPI) monitored. Embryo quality was determined using a simplified blastocyst grading system (Table 3.1.2.2-2), which has been shown to be a reliable and reproducible alternative to the Gardner system for grading embryos and assessing their prognostic potential (Richardson et al., 2015). The primary outcomes recorded were pregnancy, clinical pregnancy and live birth rates for all ETs.

Table 3.1.2.2-2 The simplified blastocyst grading system (Richardson et al., 2015)

Grade	Description
A	Expanded blastocyst, larger in size than a cleavage stage embryo with a thinning zona pellucida. Must have a clear inner cell mass and cohesive trophectoderm
B	Fully formed blastocyst with a clear inner cell mass and cohesive trophectoderm but not yet expanded with a non-thinning zona pellucida
C	Blastocyst with small/no inner cell mass and/or irregular non-continuous trophectoderm and/or excluded/degenerate cells.

3.1.2.3 Statistics

Statistical analysis was carried out with SPSS 23 (Statistical Package for Social Sciences; IBM, Chicago, IL, USA). Continuous data distribution was checked by visual inspection of histograms and a student t-test or Mann-Whitney u-test used for further analysis. All primary outcome data was categorical and assessed using a crosstabs analysis and chi-squared test. A Fisher's exact test was required only when more than 20% of categorical data outcomes were less than 5. A difference was statistically significant when $P<0.05$. A Cochran-Armitage 'test of trend' ($P <0.05$) was used for categorical outcome data to assess for an association between variables.

3.1.3 Results

188 fresh single embryo blastocyst transfers were undertaken in the 17-month period from 20th November 2014 and 29th April 2016. Clinicians adopted the two-stage after loading ET technique in 13 cases (Group M) and the VAS had not been completed in one case, so these were excluded from the eventual study group of 174 fresh single ETs. Three clinicians performed the ETs and all met the IVF centre's minimum key performance indicators, as determined by the clinical pregnancy rates expected per embryo transferred (45% ± 5%). During analysis of clinician documentation there was only one case of disagreement between experts (0.6%) when assigning difficulty groupings and the third clinician successfully resolved this. There were no significant differences in sample characteristics or blastocyst grades between groups (Table 3.1.2.3-1 and Table 3.1.2.3-2).

Table 3.1.2.3-1 The patient characteristics for both difficulty groupings

Difficulty groups	A	B	C	P-value
n=	81	38	55	
Mean Age (±SD)	34.9 (±4.4)	32.5 (±4.1)	31.9 (±3.8)	0.466
BMI (±SD)	24.0 (±2.9)	24.1 (±2.4)	24.8 (±2.3)	0.744
Non-smokers	100%	100%	100%	
Ethnicity • White • Mixed • Asian • Black	 88.9 1.2 6.2 3.7	 84.2 5.3 7.9 2.6	 85.5 1.8 10.9 1.8	
Cycle No. (±SD)	1.19 (±0.7)	1.03 (±0.2)	1.05 (±0.2)	0.580
Embryo grade % • A • B • C	 34.6 48.1 17.3	 47.4 39.4 13.2	 43.6 41.4 14.5	0.705

Table 3.1.2.3-2 The patient characteristics for VAS groupings

VAS groups	1 (<3)	2 (4-10)	3 (11-24)	4 (>24)	P-value
n=	45	43	43	43	
Mean Age (±SD)	34.7 (±3.9)	32.8 (±4.8)	33.8 (±3.8)	32.3 (±4.6)	0.531
BMI (±SD)	24.8 (±2.6)	24.2 (±3.3)	24.2 (±1.9)	25.7 (±2.3)	0.343
Non-smokers	100%	100%	100%	100%	
Ethnicity • White • Mixed • Asian • Black	 86.7 4.4 6.7 2.2	 83.7 7.2 6.8 2.3	 83.7 2.3 7.0 7.0	 81.4 2.3 14.0 2.3	
Cycle No. (±SD)	1.07 (±0.3)	1.23 (±0.8)	1.02 (±0.2)	1.12 (±0.4)	0.691
Embryo grade % • A • B • C	 37.8 44.4 17.8	 37.2 46.5 16.3	 39.5 48.8 11.6	 46.5 37.2 16.3	0.926

Table 3.1.2.3-3 Relationship between VAS scores and standard difficulty groupings (A – Easy, B – Medium, C – Difficult)

Difficulty	n =	Median	IQR	95% CI	Range
A	81	4	2-6	3-4	1-39
B	38	15	6-22	10-20	2-50
C	55	25	16-51	22-38	1-90

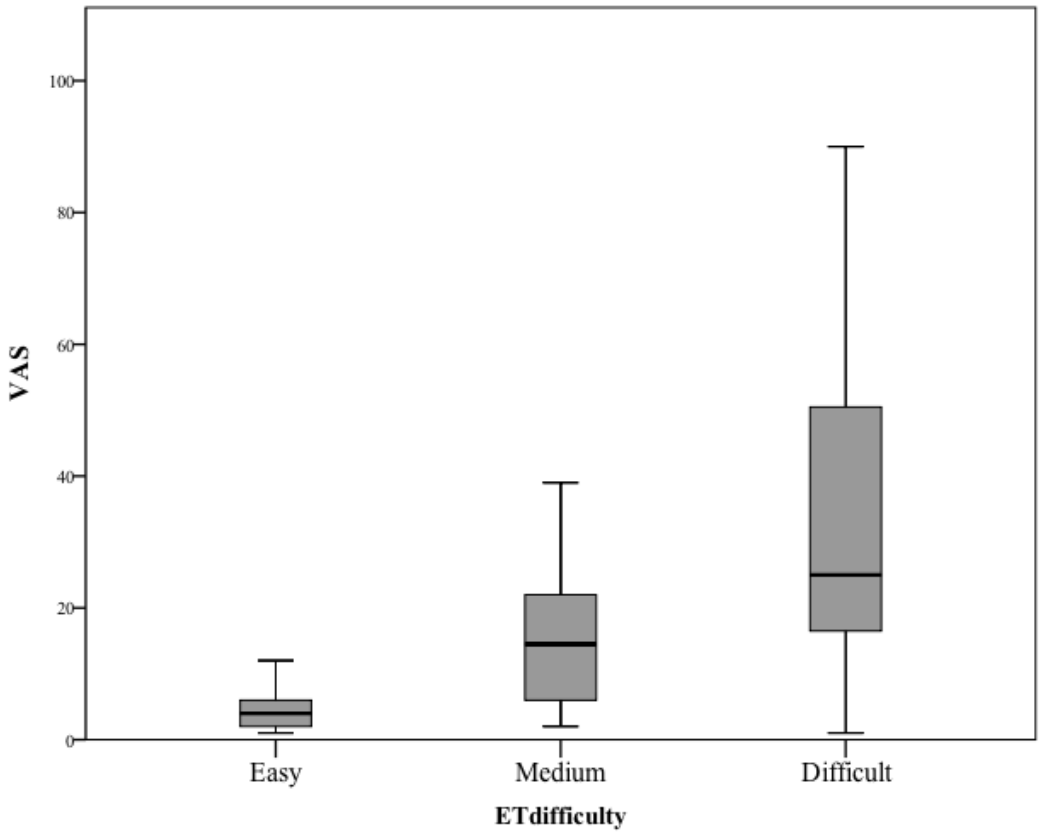


Figure 3.1.3-1 The median [-], interquartile range [solid box] and range for VAS scores in three difficulty groupings (A – Easy, B – Medium, C – Difficult)

The VAS scores in difficulty groupings A-C were skewed as demonstrated by visual inspection of histograms. Median and interquartile range values were calculated in each grouping (A-C), which demonstrated the wide variety of scores around the

median (Table 3.1.2.3-3). VAS scores in group A ranged between 1-39 with a median score of 4 (IQR 2-6, 95% CI 3-4). Group B VAS scores ranged between 2-50 with a median score of 15 (IQR 6-22, 95% CI 10-20). Group C the VAS scores ranged from 1-90 with a median score of 25 (IQR 16-51, 95% CI 22-38).

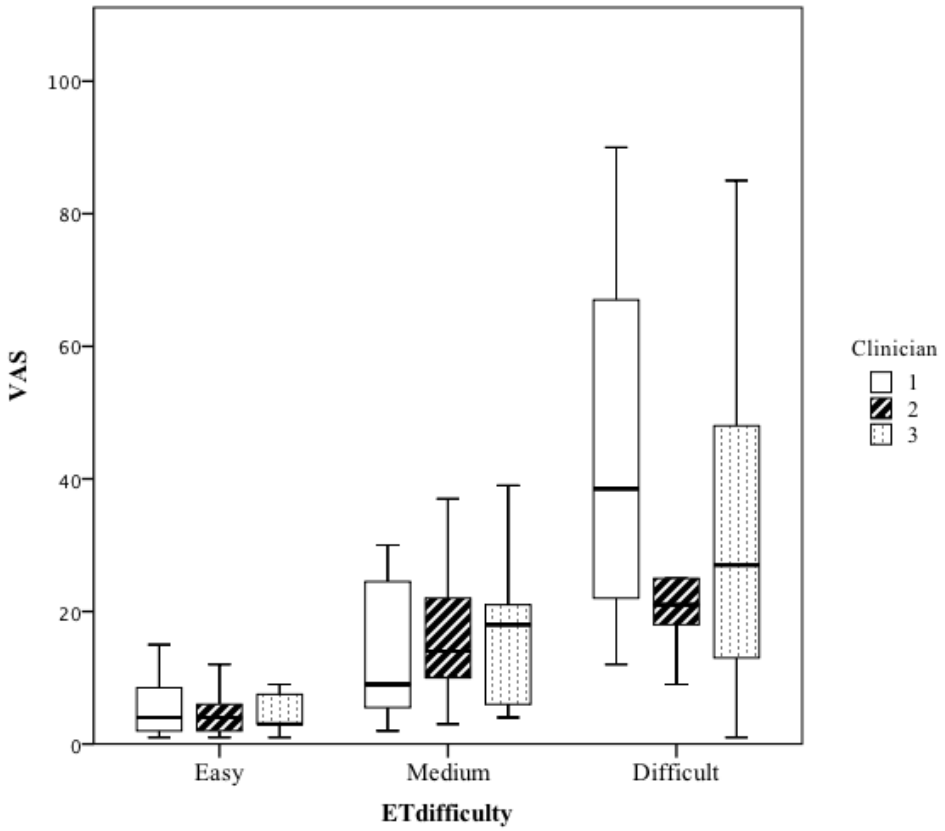


Figure 3.1.3-2 The median [-], interquartile range [solid box] and range for VAS scores in three difficulty groupings. (A – Easy, B – Medium, C – Difficult).

There was no significant relationship ($p > 0.05$) identified in pregnancy, clinical pregnancy and live birth rates in either the standard difficulty groups (

Table 3.1.2.3-4) or the VAS groupings (Table 3.1.2.3-5). The Cochran-Armitage 'test for trend' (P trend <0.05) did not reach significance for IVF outcomes in both the VAS and standard groupings, confirming this lack of association.

Table 3.1.2.3-4 IVF outcomes for both standard difficulty groupings (A-C) with risk ratios and 95% confidence intervals

Difficulty	n=	Pregnancy rate		Clinical Pregnancy		Live Birth	
		%	RR (±95% CI)	%	RR (± 95% CI)	%	RR (± 95% CI)
A	81	69.1	1	61.7	1	53.1	1
B	38	68.4	0.98 (±0.55-1.73)	60.5	0.96 (±0.60-1.57)	55.3	1.04 (±0.69-1.60)
C	55	56.4	0.71 (± 0.45-1.10)	49.1	0.75 (±0.51-1.10)	43.6	0.83 (±0.60-1.16)
P_{value}		0.272		0.313		0.450	
P_{trend}		0.140		0.157		0.310	

Table 3.1.2.3-5 IVF outcomes for both VAS difficulty groupings with risk ratios and 95% confidence intervals

Grp	VAS	n=	Pregnancy rate		Clinical Pregnancy		Live Birth	
			%	*RR (± 95% CI)*	*%*	*RR (± 95% CI)*	*%*	*RR (± 95% CI)*
1	< 3	45	62.2	1	55.6	1	46.7	1
2	4-10	43	74.4	1.48 (±0.78-2.78)	65.1	1.27 (±0.76-2.14)	55.8	1.20 (±0.78-1.86)
3	11-24	43	67.4	1.16 (±0.65-2.05)	58.1	1.06 (±0.66-1.71)	48.8	1.04 (±0.70-1.56)
4	> 24	43	55.8	0.85 (±0.52-1.41)	51.2	0.91 (±0.58-1.42)	51.2	1.09 (±0.72-1.65)
P_{value}			0.316		0.614		0.848	
P_{trend}			0.434		0.560		0.838	

3.1.4 Discussion

This is the first study to test whether the VAS tool offers a reliable alternative to the descriptive narrative used by clinicians to grade embryo transfer difficulty. These results reveal the median VAS scores allocated by clinicians at each level of difficulty (groups A-C) increased as ET became more difficult, but the interquartile ranges overlapped between groups. The broad spread of VAS scores allocated to ET procedures of similar difficulty was shown to vary greatly (Figure 3.1.3-1 and Figure 3.1.3-2) and therefore the reliability of the VAS tool to grade ET difficulty on its own is in doubt here. The wide-ranging scores allocated at each level of difficulty (A-C) suggest the scoring system is inconsistent and too reliant on the clinician's individual interpretation of what is in fact 'difficult'. As procedures became more difficult (group C) the wide confidence intervals and inter-user variability in scoring suggests clinicians found it harder to record difficulty using the VAS tool and were less consistent. Clinicians overall were more likely to record similar VAS scores when ET procedures were easier (Group A) as evidenced by the much narrower confidence intervals (Figure 3.1.3-1).

This data here suggests that the inter-user interpretation of 'difficulty' causes wide variation in clinician scoring when using the VAS tool and is not superior to clinician narrative. This is most evident when ET procedures are in fact difficult. Whilst the VAS is a simple tool that can be implemented universally in IVF clinics using different ET catheters, it provides only minimal feedback to clinicians looking to describe the ET procedure in detail. It may also be contended here that the VAS is possibly worse

than conventional narrative at recording 'difficulty', as it provides even less useful information to the clinician who is looking for the detail in the narrative that might help avoid future difficult procedures. The VAS tool is at best a simple grading tool of limited accuracy that needs further evaluation in a research setting. Clinically if the VAS is interpreted alongside descriptive narrative it may be a helpful additional tool to guide individual clinicians but caution is required.

This study showed no significant change in pregnancy, clinical pregnancy and live birth rates in either the VAS or standard difficulty groupings. Whilst these results are likely to be limited by the low numbers of transfers studied it demonstrates that neither grading system was superior to the other. Since no standardised grading system exists to measure ET difficulty this study simply adds weight to the fact that a much more robust internationally agreed system is needed to grade the difficulty of these procedures.

3.1.5 Conclusions

The ET procedure is important for IVF outcomes yet there is no internationally agreed system to grade the procedures clinicians are performing. The wide variety of ET catheters in use and subjective nature of ET 'difficulty' makes this a challenge to achieve. The VAS offers an alternative method to record procedure 'difficulty' but it has limited useful application in isolation. There is little doubt that reaching a consensus agreement and finding a widely-accepted grading system would not only

benefit future research in this area but help us to then predict more clearly when the ET procedure is likely to have a detrimental impact on implanting embryos.

3.2 TESTING A SYSTEMATIC GRADING SYSTEM ON IVF OUTCOMES

3.2.1 Introduction

The outcome of assisted reproduction treatment depends on multiple factors including the age of the patient, the presence or absence of pelvic pathology, the number of embryos transferred as well as their stage of development and quality, and whether the embryos are transferred in a fresh or frozen cycle (Balaban et al., 2000, Polanski et al., 2014, Roseboom et al., 1995). The ET itself is also important but has not been studied in as much detail (Phillips et al., 2013a).

Difficult embryo transfers have been shown to decrease pregnancy and clinical pregnancy rates (Tomas et al., 2002). Prolonged ET times, blood on the catheter and the use of a tenaculum have all been linked with difficult ET and are reported to reduce success rates (Goudas et al., 1998, Matorras et al., 2004, Schoolcraft, 2016). The higher ectopic pregnancy rates reported in IVF cycles may be in part due to the increased uterine contractility and peristalsis seen following difficult embryo transfers (Zhu et al., 2014).

It is, however, unclear what defines a ‘difficult’ transfer as there are no universally agreed classification systems and because difficulty is generally judged subjectively (Kava-Braverman et al., 2017). Although blood on the tip of the catheter and the need to use additional manoeuvres or stabilize the cervix with a tenaculum have been used as surrogate markers of difficult embryo transfer, these do not

adequately describe the wide range of techniques used or the 'lesser difficulties' often encountered in our day to day work (Akhtar et al., 2015). Furthermore, there is an inherent skill to ET that the clinician develops over years of practice that is not always immediately apparent or indeed even acknowledged by the user (Karande et al., 1999).

The number and quality of embryos replaced is also well accepted to affect pregnancy rates (Roseboom et al., 1995). The significance of these factors in the context of difficult ET is not well understood as most studies include the transfer of multiple embryos at the cleavage or blastocyst stage in both fresh and frozen cycles. The data are heterogeneous therefore and difficult to interpret. This retrospective study was designed to consider the impact of 'difficult' ET on IVF outcomes using a stratified grading scale, as assessed by pregnancy, clinical pregnancy and live birth rates. The aim being to assess the impact of ET difficulty on IVF outcomes in the context of transferring single blastocysts of variable quality.

3.2.2 Methods

All patients included in the study had IVF or ICSI treatment between June 2009 and April 2014 with a long GnRH agonist or GnRH antagonist protocol, and had a fresh day-5 single embryo transfer. Ovarian stimulation was performed with gonadotrophins and pituitary suppression with GnRH analogues (Buserilin or Cetrotide). In all cases, the human chorionic gonadotrophin (hCG) trigger used was

intramuscular Pregnyl 10,000iu or subcutaneous Ovirelle 250 micrograms and luteal support was with vaginal cyclogest pessaries.

This retrospective observational study did not require Ethics committee approval as the data collected was obtained from anonymised patient records and involved no change or intervention to standard treatment protocols. This was confirmed by the University of Nottingham ethics committee secretary by email. An 18-centimetre soft embryo replacement catheter (SureView Wallace Embryo Replacement Catheter CE118; Smiths Medical) was routinely used for all embryo transfers. The Wallace catheter consists of a flexible inner lumen that is echogenic on ultrasound. It has a stiff outer sheath that can be advanced over and beyond the tip of the inner catheter lumen to negotiate resistance during embryo replacement. This 'soft catheter' approach described here was used by all clinicians rather than the firm catheter technique that is used in some clinics. Six experienced clinicians performed ET procedures (>500 ET procedures each). All clinicians used the same stepwise approach and were audited biannually during the study period to ensure they met the IVF centres minimum key performance indicators, determined by the clinical pregnancy rates expected per embryo transferred (45% ± 5%).

3.2.2.1 The standard 'soft catheter' approach

Embryo transfers were carried out with the patient in the dorsal lithotomy position after visualising the cervix with a cusco speculum. The cervix was cleaned with normal saline and endocervical mucus removed using cotton buds. The patient had

filled their bladder prior to ET and were independently scanned by an observer with transabdominal ultrasound during the procedure to monitor the utero-cervical angle and path of the catheter. Once the embryo was loaded into the ET catheter it was inserted into the cervical os and passed through the cervix into the mid-cavity of the uterus, where the embryo was released. The embryologist gently plunged the syringe attached to the ET catheter containing 20ul of culture media and then the catheter was removed. If any resistance was met by the soft Wallace catheter clinicians had been trained to adopt a stepwise approach to overcome resistance: (1) Insert soft catheter; (2) If resistance met advance outer sheath of catheter; (3) If resistance met withdraw catheter and use firm stylet (Malleable Stylet 1816ST; Smiths Medical).

Figure 3.2.2-1 The Sure View Wallace® Embryo Replacement Catheter CE118 (Smiths Medical) combines a firm co-axial component and a soft inner lumen (pink). This was used as standard for all embryo transfer procedures adopting the 'soft-cathether approach. If clinicians were concerened about a difficult procedure the malleable stylet (blue) could be used first and moulded to assist with bypassing resistance in the cervix during ET before the soft inner catheter was passed.



3.2.2.2 The elective malleable 'dual-loading' approach

In some cases, ET was predicted to be difficult and therefore clinicians opted for an after loading technique. Embryo transfers were performed under ultrasound guidance and when clinicians anticipated a difficult ET they used a two-stage approach electively inserting a firm malleable stylet from the outset (. In these cases, after insertion of the firm malleable stylet to the level of the internal os the inner sheath was removed and the outer sheath remained. The embryologist assisting the clinician helped thread the standard soft inner catheter sheath loaded with the embryo through the outer sheath to allow the ET procedure to be completed. These cases were not included in the main analysis though due to the difference in technique used, but outcomes were considered here separately.

3.2.2.3 Developing a new 'difficulty' grading scale

A new grading scale was developed to grade the difficulty of ET procedures. All procedures were closely observed in the clinic for a two-week period. During this time, the stepwise manoeuvres made by clinicians replacing embryos was recorded and a series of steps identified. A stratified grading system to mirror the stepwise manoeuvres undertaken by clinicians replacing embryos was designed to grade each ET procedure. This difficulty grading system covers both the 'soft' catheter' (S1-10) and 'malleable stylet' (M1-6) approaches (Table 3.2.2.3-1). A 10-point grading system (S1-10) was developed to describe the 'soft-catheter' approach while a 6-point grading scale (M1-6) describes the 'dual loading' technique.

The S1-10 scale is based on three principal manoeuvres (A-C) made by clinicians with the catheter to overcome resistance as was described previously in Table 3.1.2.2-1. It was designed to capture the subtle movements made during an ET procedure, as these lesser difficulties have not always been well recognised in previous difficulty grading systems (Table 2.1.2.5-1). However, it was also designed to provide a graduated stepwise scale of increasing difficulty based on routine manoeuvres. The 10-point grading system records both the 'lesser difficulties' that clinicians experience but also records the more commonly reported manoeuvres associated with 'higher levels of difficulty', such as use of a firm stylet or a tenaculum. Additional degrees of 'difficulty' were added at each level based on levels of resistance and how easy it is to perform that manouevere and bypass this resistance. The different levels of this new grading system are described here:

(A) Advance soft inner (S1 - S2)

This level subdivided into two further degrees of difficulty (S1 and S2) based on the amount of resistance and bend in the catheter, though the catheter still passes without the need for additional manoeuvres.

(B) Advance co-axial sheath (S3-S5)

This level is subdivided into three further degrees of difficulty based on how easy it is to advance the co-axial sheath. If the co-axial sheath is advanced and the catheter passes straight away this is considered easy (S3), if there is some resistance but after a short delay and a few simple adjustments the catheter passes this is regarded to

have involved ‘some difficulty’ (S4) and is it takes a lot of longer and resistance is high but the coaxial eventually passes then it is considered ‘difficult’ (S5)

(C) Resort to a firm malleable stylet (S6-S9)

This level is subdivided into four further degrees of difficulty based on how easy it is to overcome resistance with the firm stylet after failing with the co-axial sheath. If the stylet is advanced and it passes straight away with minimal resistance this is considered ‘easy’ (S6). If there is some resistance but after a short delay and a few simple adjustments the stylet passes this is regarded to have involved ‘some difficulty’ (S7). If resistance is very high but the stylet eventually passes then it is considered ‘difficult’ (S8). Included is the use of a tenaculum for counter-traction in this level as the stylet is still being used at this point (S9).

(D) Unable to access

If access was not possible due to resistance levels being too high then this type of ET procedure was labelled S10.

(M) Elective Malleable

A clinician with concerns about a potentially difficult ET procedure was trained to use the ‘elective malleable’ dual-loading approach. This approach was subdivided into different levels of difficulty based on the resistance encountered (M1-5) as the firm malleable stylet passes (Table 3.2.2.3-1).

Table 3.2.2.3-1 The stepwise increase in difficulty levels based on both the standard 'soft catheter' approach (S1-10) and the firmer 'elective' malleable (M1-6) approach used to replace an embryo. The different categories of difficulty are described in detail; (A) S1-2 – Easy, (B) S3-5 – Medium, (C) S6-9 – Difficult, (M) M1-5 – Malleable

		Standard Approach (S) – 'soft catheter'	
Easy	**A**	**S1**	Easy / no resistance / no bend in catheter
		S2	Easy / some resistance / bend in catheter
Medium	**B**	**S3**	Resistance / outer advanced / easy
		S4	Resistance / outer advanced / some difficulty
		S5	Resistance / outer advanced / difficult
Difficult	**C**	**S6**	Resistance / resort to malleable / easy
		S7	Resistance / resort to malleable / some difficulty
		S8	Resistance / resort to malleable / difficult
		S9	Resistance / resort to malleable / extra manoeuvres
	D	**S10**	Unable to gain access
		Elective Malleable (M) – 'dual-loading'	
Malleable	**M**	**M1**	Malleable / easy / no resistance
		M2	Malleable / minimal resistance
		M3	Malleable / some difficulty
		M4	Malleable / difficult
		M5	Malleable / difficult / extra manoeuvres
		M6	Unable to gain access

3.2.2.4 Data collection

The ET procedure itself was systematically recorded on a standard proforma with attention paid to the stepwise approach taken to replace the embryo. Clinicians recorded: resistance to the catheter and use of the outer co-axial sheath; the need to resort to a malleable stylet; and tenaculum use. Three different levels of difficulty were identified and stratified objectively (Table 3.1.2.2-1) according to the clear steps taken by the clinician to overcome resistance.

Each ET documentation record was scrutinised by two observers (EC and HC) independently and the manoeuvres that were reported were stratified to determine how difficult the transfer had been. When there was disagreement a third observer (NRF) was asked to categorise the outcome based on the documentation. Rates of blood on the catheter and tenaculum usage were collected for each grouping. Patient characteristics (age, BMI, ethnicity, smoking, and cycle number) were collated (Table 3.2.2.4-1), and clinician key performance indicator (KPI) for pregnancy and clinical pregnancy rates were recorded. Embryo quality was determined using a simplified blastocyst grading system (Table 3.1.2.2-2) which has been shown to be a reliable and reproducible alternative to the Gardner system for grading embryos and assessing their prognostic potential (Richardson et al., 2015, Richardson et al., 2016).

The main criteria for inclusion in the study was that the transfer procedure must have involved the replacement of a single fresh embryo at the blastocyst stage.

Excluded from the study were any treatment cycles that involved the replacement of multiple embryos, frozen embryos or embryos at the cleavage stage. Primary clinical outcomes measured were pregnancy, clinical pregnancy and live birth rates. The pregnancy rate was defined as a positive urinary pregnancy test on day 13 following blastocyst transfer. Clinical pregnancy was defined as a fetal heartbeat seen on ultrasound at six weeks' gestation and live birth as the birth of a live baby after 24 weeks' gestation.

Table 3.2.2.4-1 Patient characteristics for ET difficulty groupings and embryo grade

ET difficulty	**Easy**	**Medium**	**Difficult**	***Malleable**	**P-value**
n =	385	119	148	86	
Age	33.57 ± 4.71	32.82 ± 3.86	32.78 ± 5.52	33.51 ± 5.29	0.239
BMI	24.41 ± 3.17	24.09 ± 2.88	24.24 ± 3.21	24.51 ± 4.07	0.738
Non-smokers (%)	100	100	100	100	-
Ethnicity (%) • White • Mixed • Asian • Black	 85.19 5.97 7.79 1.04	 80.67 7.56 8.40 3.36	 74.32 6.76 15.54 3.38	 82.56 3.49 13.95 0.00	-
Mean Cycle No.	1.23 ± 0.71	1.18 ± 0.67	1.11 ± 0.33	1.49 ± 1.30	0.002
Blood	12/385	7/119	10/148	7/86	< 0.001*
Tenaculum (%)	0	1/119	0	0	0.182
Embryo Grade	**Grade A**	**Grade B**	**Grade C**		
n =	299	298	141		
Age	33.3 ± 4.4	33.1 ± 5.3	33.8 ± 4.7		0.334
BMI	24.1 ± 3.3	24.2 ± 3.1	25.0 ± 3.2		0.017*
Non-smokers (%)	100	100	100		-
Ethnicity (%) • White • Mixed • Asian • Black	 78.73 5.97 12.31 2.99	 83.34 6.06 9.09 1.52	 85.00 10.00 5.00 0.00		-
Mean Cycle No.	1.21 ± 0.7	1.19 ± 0.6	1.34 ± 1.0		0.130
Blood	16/299	10/298	8/141		0.668
Tenaculum	0	1/298	0		0.480

3.2.2.5 Statistical analysis

Statistical analysis was carried out using SPSS 23 (Statistical Package for Social Sciences; IBM, Chicago, IL, USA). All continuous variables were checked for normal distribution by visual inspection of histograms and a student t-test or Mann-Whitney u-test if required. A one-way analysis of variance (ANOVA) was performed to check for significance of cycle characteristics across the groups. All primary outcome data were categorical and assessed initially using a crosstabs analysis from which chi-squared values were calculated. A Fisher's exact test was not required in this data analysis as none of categorical data outcomes were less than 5. A multiple logistic regression analysis was performed adjusting for 'a priori' and cycle characteristic confounders. A difference was statistically significant when $P<0.05$.

3.2.3 Results

754 fresh single embryo transfers were performed over a period extending from June 2009 and April 2014. The ET procedure records were obtained and found to be incomplete in 16 cases (2.1%), so these had to be excluded from the analysis. There was disagreement in determining the difficulty classification in only two cases and both were resolved easily by a third clinician (NRF). Two study groups were formed and outcomes analysed based on the ET technique. Clinicians had opted to pass the malleable stylet electively in 86 cases (group M), using a 'dual loading' technique and

so these outcomes were initially excluded from the first logistic regression analysis (Table 3.2.3.4-1) but included in a second logistic regression model.

A logistic regression model of ET 'difficulty' including the 'elective malleable' outcome data was evaluated (Table 3.2.3.4-2). A significant fall in pregnancy rates was seen for Grade A embryos (p = 0.011) as difficulty increased, but lower quality blastocysts (grade B and C) did not show the same significant fall. A good quality (Grade A) embryo's chance of pregnancy fell by 68% with a 'difficult' transfer (OR0.32, ± 0.17-0.63) compared to an 'easy' transfer'.

It is of interest that if the blastocyst was transferred using the 'elective malleable' technique, and avoided a 'difficult' ET, the chances of pregnancy fell by only 40% (OR 0.60, ± 0.23-1.52) in comparison. Similarly, the grade C blastocysts appeared to benefit from the elective malleable technique with pregnancy chances improving compared to 'difficult' ET though these differences were not significant (p = 0.114).

652 single blastocyst transfers formed the study group in the initial analysis, excluding 'elective malleable' procedures (Group M), and there were no significant differences in patient characteristics across the groupings. 738 patients formed the study group when the elective malleable procedures were included and they were noted to have more blood on the ET catheter and a higher mean cycle number overall, so these factors were adjusted for in the logistic regression model (Table 3.2.2.4-1).

3.2.3.1 For all embryos

Without differentiating for embryo grade, there was a significant fall in both pregnancy and clinical pregnancy rate when the transfer procedure became more difficult, but there was no effect on live birth. Pregnancy rates fell from 67.8% with an easy transfer to 62.2% and 56.1% with medium and difficult transfers respectively (p=0.037). Clinical pregnancy rates showed a similarly significant difference (p = 0.037) as rates dropped from 59.0% for an easy transfer to 46.4% for a difficult transfer. Live birth rates fell from 51.4% to 43.2% when an ET was difficult but this difference was not significant (p = 0.202) (Table 3.2.3.2-1).

3.2.3.2 Based on embryo quality

The pregnancy rates (PR) fell from 84.3% to 62.0% for the highest quality (grade A) embryos as the transfer became difficult (p = 0.001) (Table 3.2.3.2-1). Whilst this fall in pregnancy rates was significant for the best quality embryos, which have the highest prognostic potential, it was not the same for the lower quality grade B and C embryos. Clinical pregnancy (CP) rates fell for all grades of embryo as transfer difficulty increased. Despite this clear fall in CP and LB rates and a noticeable effect the results did not reach statistical significance ($P < 0.05$).

*Table 3.2.3.2-1 Pregnancy, Clinical Pregnancy and Live Birth rates according to blastocyst quality (Grade A-C) and difficulty of ET (*malleable ET data not included in statistical analysis)*

	n =	Pregnancy (%)	Clinical pregnancy (%)	Live Birth (%)
ALL				
Easy	385	67.8	59.0	51.4
Medium	119	62.2	54.6	52.1
Difficult	148	56.1	46.6	43.2
	(652)	*p = 0.037*	*p = 0.037*	*p = 0.202*
Grade A				
Easy	140	84.3	72.9	65.0
Medium	56	75.4	68.4	64.9
Difficult	71	62.0	57.7	54.9
	(267)	*p = 0.001*	*p = 0.084*	*p = 0.326*
**(Malleable)*	*32*	*75.0*	*68.8*	*62.5*
Grade B				
Easy	169	66.9	61.5	53.3
Medium	39	60.5	52.6	50.0
Difficult	56	64.9	45.6	40.4
	(264)	*p = 0.754*	*p = 0.094*	*p = 0.242*
**(Malleable)*	*34*	*52.9*	*41.2*	*32.4*
Grade C				
Easy	76	39.5	27.6	22.4
Medium	24	33.3	25.0	25.0
Difficult	20	10.0	10.0	10.0
	(121)	*p = 0.045*	*p = 0.260*	*p = 0.410*
**(Malleable)*	*20*	*35.0*	*25.0*	*25.0*

3.2.3.3 Elective malleable

The elective malleable approach (Group M) was used by clinicians in 86 cases when a difficult procedure was anticipated and accounted for 11.7% of all single blastocysts transfers. Although 'difficulty' was expected it did not always transpire, with 62 of these cases (72%) recorded as being 'easy without resistance' (M1 - Table 3.2.2.3-1). Whilst only a small number of embryos were transferred in this way, the poorer quality embryos (Grade C) appear to benefit from the elective malleable approach with pregnancy rates rising from 10% with a difficult transfer to 35%, which was closer to that attained with an easy transfer (39.5%). Further analysis in a logistic regression model is needed to evaluate these outcomes and adjust for confounders.

3.2.3.4 Interaction of ET difficulty and embryo quality in a regression model

The impact of ET 'difficulty' on pregnancy and clinical pregnancy rates in the context of changing embryo quality was examined further with a multivariate analysis using logistic regression (Table 3.2.3.4-1). Analysing IVF outcomes in a single statistical model adjusting for covariates (Age, BMI, cycle number, blood on the catheter), showed a fall in pregnancy rates for good quality (Grade A) embryos ($p = 0.004$) as difficulty increased from 'Easy' to 'difficult'. There was a fall in pregnancy rates for lower quality embryos (Grade B and C), but this did not reach statistical significance. Clinical pregnancy rates fell as difficulty increased but for all embryo of differing quality this did not reach statistical significance.

Table 3.2.3.4-1 Effect of ET 'difficulty' on pregnancy and clinical pregnancy rates according to embryo quality (Grade A-C) with odds ratios adjusted for covariates ± 95% CI in a logistic regression model.

		Pregnancy		Clinical Pregnancy	
Grade A	n	**OR (adj)**	**± 95% CI**	**OR (adj)**	**± 95% CI**
Easy	140	1		1	
Medium	56	0.61	0.28-1.34	0.85	0.43-1.69
Difficult	71	0.32	0.17-0.63	0.51	0.28-0.94
	(267)	*P = 0.004*		*P = 0.091*	
Grade B					
Easy	169	1		1	
Medium	39	0.71	0.35-1.45	0.65	0.32-1.31
Difficult	56	0.96	0.51-1.82	0.54	0.29-0.99
	(264)	*P = 0.637*		*P = 0.105*	
Grade C					
Easy	76	1		1	
Medium	24	0.80	0.30-2.12	0.89	0.31-2.55
Difficult	21	0.15	0.03-0.71	0.28	0.60-1.30
	(121)	P = 0.057		*P = 0.262*	

A logistic regression model of ET 'difficulty' including the 'elective malleable' outcome data was evaluated (Table 3.2.3.4-2). A significant fall in pregnancy rates was seen for Grade A embryos (p = 0.011) as difficulty increased, but lower quality blastocysts (grade B and C) did not show the same significant fall. A good quality (Grade A) embryo's chance of pregnancy fell by 68% with a 'difficult' transfer (OR0.32, ± 0.17-0.63) compared to an 'easy' transfer'.

It is of interest that if the blastocyst was transferred using the 'elective malleable' technique, and avoided a 'difficult' ET, the chances of pregnancy fell by only 40% (OR 0.60, ± 0.23-1.52) in comparison. Similarly, the grade C blastocysts appeared to

benefit from the elective malleable technique with pregnancy chances improving compared to 'difficult' ET though these differences were not significant (p = 0.114).

Table 3.2.3.4-2 Effect of ET 'difficulty' on pregnancy and clinical pregnancy rates with odds ratios adjusted for covariates ± 95% CI (+ malleable) in a logistic regression model

		Pregnancy		**Clinical Pregnancy**	
Grade A	n	**OR (adj)**	**± 95% CI**	**OR (adj)**	**± 95% CI**
Easy	140	1		1	
Medium	56	0.63	0.29-1.37	0.88	0.44-1.74
Difficult	71	0.32	0.17-0.63	0.52	0.28-0.95
Malleable	32	0.60	0.23-1.52	0.86	0.37-2.01
	(299)	*P = 0.011*		*P = 0.199*	
Grade B					
Easy	169	1		1	
Medium	39	0.69	0.34-1.42	0.65	0.32-1.32
Difficult	56	0.94	0.50-1.79	0.54	0.30-1.00
Malleable	34	0.58	0.27-1.23	0.45	0.21-0.96
	(298)	*P = 0.444*		*0.071*	
Grade C					
Easy	76	1		1	
Medium	24	0.80	0.30-2.14	0.90	0.31-2.60
Difficult	21	0.15	0.31-0.69	0.27	0.06-1.25
Malleable	20	0.91	0.31-2.67	0.94	0.30-3.00
	(141)	*P = 0.114*		*P = 0.417*	

3.2.4 Discussion

This is the first study to look at how ET difficulty affects IVF outcome in the context of the replacement of a single blastocyst. It is also the first study to consider embryo

quality in this way. In previous studies, transfer difficulty has been studied in cycles replacing multiple embryos of differing qualities. The true impact of difficult ET on an embryo's prognostic potential is more accurately assessed in single embryo replacement cycles.

These findings agree with previous studies that have demonstrated an embryo's potential to implant and form a clinical pregnancy is significantly reduced when the transfer of that embryo is 'difficult'. This study shows that single blastocysts transferred in a soft catheter have significantly higher Pregnancy and clinical pregnancy rates when the procedure has been easy. It is of clinical interest that live birth rates fell from 51.4% to 43.2 % with the most difficult transfers, although this did not reach scientific significance ($p = 0.202$). In an age of 'marginal gains' a fall in live birth rates to this degree deserves attention and further work.

This data has also shown, for the first time, that a difficult ET appears to have the greatest impact on the highest quality embryos which have the greatest potential to succeed. This study showed a difficult ET, as defined by a failure to overcome resistance with the outer sheath requiring the use of a malleable stylet, significantly reduces Pregnancy rates when a top-quality blastocyst (Grade A) is replaced. The effect of a difficult ET seems to be most significant for the highest quality blastocysts (Grade A) compared to poorer quality blastocysts (Grade B & C) which have lower potential to implant.

There are limitations to this study as the ET 'difficulty' classification relies on the interpretation and reporting of the steps taken by the clinician to replace the embryo. The stratification system used in this study (Table 3.2.2.3-1) accounts for the 'soft catheter' approach (S1-10) and the 'dual-loading' technique (M1-6) which covers most techniques. Whilst these difficulty levels are based on the Wallace SureView catheter and may not apply to all clinics where different ET catheters and techniques are used, the approach stratifies ET 'difficulty' in a way that is more robust than many studies in the literature. As in many other studies no automated embryo grading system yet exists and therefore a grade was allocated subjectively to each embryo by the embryologist, which may also be open to inter-user variation.

Research into the subjective interpretation of how difficult an embryo transfer has been and its effect on IVF outcomes remains a challenge and the focus of this thesis. A much larger prospective study using this stratified ET 'difficulty' grading system is needed to explore whether the prognostic potential of a single blastocyst is changed significantly by difficult ET. If, as this study suggests though, a difficult ET is more detrimental to the prognostic potential of higher quality embryos, then further work is needed to understand the mechanism and to avoid difficult transfers in this group.

One of the problems in clinical practice is that it is challenging to predict which patients will experience a difficult embryo transfer. It is possible that the alternative method of inserting a malleable stylet into the cervix ahead of transfer, as was used in 86 cases here, may represent a more appropriate approach to replace embryos when a difficult transfer is expected. However, the answer to this will require both

techniques to be compared in a non-inferiority, randomised controlled trial, and this can only be done once a system exists to accurately predict difficult ET in our patients. Further work is needed here around ET difficulty to interpret how critical embryo replacement is on IVF outcomes in the context of embryo quality and single embryo transfer.

3.2.5 Conclusion

This study has shown that a difficult ET has a significant impact on the potential of a single high quality (Grade A) blastocyst to implant. This fall in prognostic potential affects all embryos to some degree but the highest quality blastocysts seem most affected. Further work focusing on high quality embryos will help to determine the key factors that affect implantation. Factors such as the time taken to complete the transfer, the presence of blood during transfer, and the exposure of embryos to environmental stressors such as changes in pCO_2 and culture medium acidity have all been considered important (Cohen et al., 1997, Alvero et al., 2003, Matorras et al., 2004, Ciray et al., 2007).

Chapter 4

Embryo transfer time and IVF outcomes

4.1 THE IMPACT OF TIME EXPOSURE ON EMBRYO POTENTIAL

4.1.1 Introduction

It was established in 'Chapter 2' that the different descriptive systems for reporting ET 'difficulty' are not the same across the literature and when analysed systematically high levels of heterogeneity were found in studies reporting the prevalence of 'difficult' ET. Some authors had reported the prevalence of ET 'difficulty' as 0.4% (Matorras et al., 2002) and 3.8% (Spitzer et al., 2012), whilst others have reported levels of difficulty as high as 18.5% (Ghaffari et al., 2013) and 29.5% (Tiboni et al., 2012b). Whilst it is accepted that difficult ET is an independent prognostic factor in determining IVF outcomes, the prevalence of 'difficulty' varies considerably across the literature due to the multitude of different classification systems used by clinicians.

Clinicians frequently use surrogate markers of difficulty such as; the need to dilate the cervix, the presence of blood on the catheter, the use of a firm catheter or the use of a tenaculum. Whilst some investigators have relied upon single usage of these terms others have grouped them together when defining whether an ET procedure has been difficult. Many of these markers of 'difficulty' are important but there are potentially alternative systems available that are more sensitive and user-friendly and perhaps more directly relevant when assessing ET difficulty and outcomes. Many of the lesser manoeuvres made by the hands of a clinician manipulating the catheter, as was shown in 'chapter 3', are also important affecting pregnancy

outcomes. Despite the many studies looking at ET 'difficulty' there is still no refined understanding of what it is exactly that is reducing the embryo's potential to implant.

It is not unusual for increasingly difficult ET procedures to be more time-consuming dependent on the technique used by the clinician. The clinician can either adopt the standard technique and load the embryos into a soft catheter and replace them directly into the uterus. However, some clinicians prefer to use a dual-loading technique and perform the ET in two stages. The dual loading technique involves first positioning a soft catheter with its outer sheath advanced as far as the internal cervical os, or alternatively using a firmer malleable stylet to achieve the same effect bypassing any resistance in the cervix. In both these cases the inner section of the catheter is removed and the embryos, which have been loaded separately into a soft inner catheter, pass through the outer sleeve into the correct position in the uterus.

The ETT is also referred to as the interval between loading the embryos into the catheter and discharging them into the uterus. The ETT is a continuous dependent variable that relates to the length of time the embryos remain inside the catheter and this measurement relies on the stopping and starting of a timer. During the ETT, the embryos are exposed in the catheter to room temperature and light for variable lengths of time. There have been two studies that have looked at ETT and explored how it impacts on IVF outcomes, as it has been unclear whether prolonged transfers may be detrimental to an embryo's prognostic potential to implant.

Matorras et al 2004 concluded that the ETT was an independent factor affecting implantation and clinical pregnancy rates. In that study 3-4 fresh embryos were transferred at the cleavage stage and the ETT recorded. Four groups according to ETT were established and the study showed a significant fall in pregnancy and clinical pregnancy rates as time increased. The highest pregnancy and pregnancy rates were seen when ETT was <30 seconds and there was a downwards trend which fell sharply once ETT reached 120 seconds. This effect was independent of ET 'difficulty' as the same effect was seen when adjusting for the 'difficulty' of ET. Ciray et al 2007 also studied the effect of ETT when transferring 2-3 fresh cleavage stage embryos and they attempted to control for both 'embryo quality' and ET 'difficulty'. Outcomes were assessed in four groups according to ETT: (1) 0-60 seconds, (2) 61-120 seconds, (3) 121-180 seconds, (4) >180 seconds. Ciray's findings contradicted the work by Matorras, showing no effect of ETT on pregnancy of clinical pregnancy success (Matorras et al., 2004, Ciray et al., 2007).

Each of these studies used different criteria to define 'difficult' ET and this could explain the differences between the outcomes. Mattoras et al 2004 regarded 'difficult' ET as when a tenaculum had been applied to the cervix. Ciray et al. 2007 heavily criticised this definition but then did not clearly describe the criteria they used in their own study, though they did state that no tenaculum had been applied to the cervix in any ET procedures. Both studies replaced multiple fresh embryos at the cleavage stage in each patient and demonstrated that difficult ET resulted in prolonged transfer times. Ciray et al 2007 had attempted to control for embryo quality but were limited by the fact that the number of transfers were too small to

be able to draw any meaningful conclusion. As a result, there are two studies in the literature that contradict each other but both address a really important issue.

It is not known if ETT is an independent factor affecting the pregnancy and clinical pregnancy rates in an IVF cycle, and the importance of this in the context of controlling for embryo quality has still not been answered. This study set out to find out whether ETT affects pregnancy and clinical pregnancy rates in single blastocyst transfer cycles when you control for embryo quality. ET 'difficulty' was not measured in this study, simply because 'chapter 2' identified marked heterogeneity and disagreement on how to define 'difficulty'. However, this study did measure the important surrogate markers of 'difficulty', such as use of a tenaculum and blood on the catheter as these have been identified as potential confounders in the studies by Matorras and Ciray.

4.1.2 Materials and methods

4.1.2.1 Methods

The aim of this study was to examine the effects of the ETT on pregnancy and clinical pregnancy rates in the context of transferring single fresh blastocysts of variable quality. In March 2015, the IVF clinic (Nurture fertility) moved to a purpose-built facility with a new laboratory and ET theatre, so this was an ideal time to begin this next phase of experimentation. No additional research ethics approval was required to measure the ETT as this was confirmed byt the research ethics committee to be a

change in service provision, and so recording of ETT for all ET procedures was therefore introduced into routine practice.

Patients included in the study group had undergone IVF or ICSI long GnRH agonist or short GnRH antagonist treatment cycles that resulted in the transfer of a fresh single blastocyst. Ovarian stimulation was with gonadotrophins and pituitary suppression with GnRH analogues (Buserilin or Cetrotide). In all cases, the human chorionic gonadotrophin (hCG) trigger used was intramuscular Pregnyl 10,000iu and luteal support was with cyclogest pessaries. Excluded from the study group were any patients undergoing donor egg cycles.

Important patient and cycle characteristics (age, AMH level, endometrial thickness, oocyte collection day, peak E2, cycle number, number of blastocysts) were also collected for consideration in This analysis. All ET procedures were either performed with the standard soft catheter approach as described in 3.2.2.1 or the elective malleable 'two-stage' approach (3.2.2.2). Surrogate markers of difficulty such as blood on the ET catheter or use of a tenaculum were recorded. Embryo quality was once again determined using the simplified blastocyst grading system (Table 3.1.2.2-2) which is a reliable and reproducible alternative to the Gardner system for grading and assessing an embryos prognostic potential (Richardson et al., 2015, Richardson et al., 2016). Only those patients undergoing the replacement of a single grade A or grade B blastocyst were inlcuded and the ETT data was analysed both collectively and in sub-groups according to embryo grade. Primary outcomes were pregnancy and clinical pregnancy rates as defined by a positive urinary pregnancy

test on day 13 after embryo transfer, and clinical pregnancy was defined as a fetal heart seen on ultrasound scan at 7 weeks' gestation.

4.1.2.2 Definition of ETT

All ET procedures took place in the same ET theatre adjoining the main embryology laboratory at Nurture, and therefore once the embryo has been loaded from the ET dish between two air bubbles into the SureView Wallace catheter it is carried through the adjoining door into the ET theatre for the clinician to transfer the embryo. The ET catheter (SureView Wallace, Smiths Medical) is pre-warmed in the laminar flow hood (IVF tech) prior to loading. The patient is positioned in the lithotomy position and the clinician attains a 360-degree view of the cervix and cleans any visible mucus. The clinician makes their final decision about using the standard or malleable (dual-loading) technique and begins the procedure. At this point on the instructions of the clinician the embryologist removes the ET dish from the MINC incubator and rests it on the warmed plate in the ET laminar flow hood (IVF tech), and begins embryo loading.

Prior to each ET procedure the embryo has been pre-prepared in a single ET dish containing 1ml of G2 culture media (Vitrolife, Sweden) and this remains in the MINC desktop incubator next to the laminar flow hood. The ETT begins when the catheter has been successfully loaded with an embryo and the ETT ends once the ET procedure is complete and the embryologist has checked the ET catheter is empty. The embryologist's skill level and speed loading embryos was an important factor to

consider. The average time from removing the ET dish from the MINC incubator to successfully loading the catheter over a series of ET procedures was recorded for each embryologist, to check for agreement between embryologists in terms of loading-time. This was important as when the ET dish leaves the incubator the embryo sits in 1ml of G2 media and is exposed to environmental conditions

Mattoras et al (2004) had suggested that '120 seconds' was the time at which a significant drop in implantation rates was noticed. Ciray et al (2007) looked at the ETT in four groups but found no difference in implantation rates. Neither study outlined in detail how the ETT had been measured, as has been done here. The best way to test the significance of the '120-second' ET window on pregnancy outcomes was to establish three different ETT models, categorising time into increments spanning this potentially important cut-off point.

4.1.2.3 ETT groupings for multivariate analysis

The impact of ETT and embryo quality on pregnancy and clinical pregnancy rates was analysed using a multivariate analysis (see 4.1.2.4 and an assessment made as to whether there was an interaction between these two independent variables. The effect of ETT on pregnancy and clinical pregnancy rates was considered in four different binary logistic regression models: (1) Six ETT groups, (2) Three ETT groups, (3) Two ETT groups and (4) Continuous ETT. These groupings were defined and based on the previous evidence from Matorras and Ciray et al. that "120-seconds" was an important cut-off point at which success rates change (Table 4.1.2.3-1).

Table 4.1.2.3-1 ETT groupings (seconds) for multivariate analysis

Model	ETT Groups	1	2	3	4	5	6
1	*6-groups*	< 90	90-119	120-149	150-179	180-240	240+
2	*3-groups*	< 120	120-179	180+			
3	*2-groups*	< 120	> 120				
4	*Continuous*						

4.1.2.4 Statistics

Statistical analysis was carried out with SPSS 23 (Statistical Package for Social Sciences; IBM, Chicago, IL, USA). Continuous data distribution was checked by visual inspection of histograms and a student t-test or Mann-Whitney u-test used for further analysis. All primary outcome data was categorical and assessed using crosstabs analysis and chi-squared test of significance. A Fisher's exact test was required when more than 20% of categorical data outcomes were less than 5. A difference was statistically significant when $P<0.05$. A multivariate analysis was undertaken using binary logistic regression calculating odds ratios and confidence intervals. Negelkerke R_2 and Hosmer and lemeshow values were calculated to assess the fit of the logistic model to the data.

4.1.3 Results

4.1.3.1 For all embryos

734 cases were included in the study group. There were no significant differences in patient and cycle characteristics between the ETT categories (Table 4.1.3.1-1).

*Table 4.1.3.1-1 Patient and cycle characteristics based on ETT and embryo grade groupings – mean values (± 1 SD) unless specified, [*Embryo Grade 1= grade A blastocyst and 2= grade B blastocyst]*

Variable / ETT groups	< 90 secs	90-119 secs	120-149 secs	150-179 secs	180-239 secs	> 240 secs	P value
n=	152	239	148	50	73	71	
Age (yrs)	33.0 (± 4.1)	32.9 (± 4.4)	32.8 (± 3.8)	32.0 (± 4.4)	32.5 (± 4.3)	31.4 (± 4.0)	0.082
Non-smokers	100%	100%	100%	100%	100%	100%	-
Mean Cycle No.	1.29 (± 1.01)	1.23 (± 0.72)	1.16 (± 0.51)	1.12 (± 0.33)	1.08 (± 0.40)	1.17 (± 0.61)	0.281
AMH	23.4 (± 16.6)	22.6 (± 16.0)	24.9 (± 18.6)	25.4 (± 16.0)	21.6 (± 14.2)	24.5 (± 14.0)	0.652
Egg collection day	14.3 (± 1.5)	14.4 (± 1.6)	14.0 (± 1.4)	14.1 (± 1.1)	14.3 (± 1.6)	14.2 (± 1.4)	0.135
Endometrial thickness (mm)	11.0 (± 2.6)	10.6 (± 2.3)	10.4 (± 2.2)	11.1 (± 2.4)	11.6 (±2.7)	11.3 (± 1.8)	0.156
Peak E2	6779 (± 3982)	7238 (± 3789)	6989 (± 3928)	9693 (± 3933)	7173 (± 3406)	7508 (± 3813)	0.183
Blastocysts available	4.9 (± 2.8)	4.9 (± 3.2)	4.9 (± 3.3)	5.0 (± 3.2)	5.0 (± 3.1)	5.2 (± 3.4)	0.992
Embryo grade*	1.36 (± 0.48)	1.43 (± 0.50)	1.42 (± 0.50)	1.40 (± 0.50)	1.45 (± 0.50)	1.44 (± 0.50)	0.689
Blood on catheter %	2.0	0.8	0.6	4.0	4.0	9.9	0.062
Tenaculum %	0	0	0	0	1.4	2.8	0.432

Variable / Embryo Grade	Grade A	Grade B	P value
n=	431	303	
Age	32.6 (± 4.3)	32.6 (± 4.0)	0.994
Non-smokers	100%	100%	-
Mean Cycle No.	1.19 (± 0.70)	1.21 (± 0.71)	0.737
AMH	24.3 (± 16.2)	22.4 (± 16.6)	0.156
Egg collection day	14.1 (± 1.6)	14.4 (± 1.4)	0.517
Endometrial thickness (mm)	10.8 (± 2.4)	11.1 (± 2.4)	0.190
Peak E2	7415 (± 3992)	6861 (± 3586)	0.214
Blastocysts available	6.0 (± 3.3)	3.6 (± 5)	<0.001
Blood on catheter %	1.9	3.3	0.265
Tenaculum %	0.5	0.3	0.852

Inspection of histograms showed good evidence of normally distributed data. A multivariate analysis with adjusted odds ratios accounting for covariates (age, AMH, mean cycle number, peak E2, embryo grade, endometrial thickness, days to egg collection, blastocyst number) showed a fall in pregnancy and clinical pregnancy rates.

The ETT as defined earlier in 4.1.2.2 consisted of the time from successful embryo loading of the catheter to completion of the ET procedure with confirmation that no embryo remained in the catheter. The average embryologist loading time was 41 seconds (± 4.8 secs). The longest time taken to load the catheter was 50 seconds and the shortest time to load the embryo was 32 seconds.

IVF outcomes were initially considered with ETT as a continuous variable in a binary logistic regression model. This revealed a significant fall in pregnancy rates per second of time increase in ETT ($p = 0.045$), and the Hosmer lemeshow goodness of fit was 0.820 suggesting a good fit between the model and the data. In a similar model, clinical pregnancy rates also fell but they did not quite reach significance ($p = 0.057$). The impact of ETT on pregnancy and clinical pregnancy rates was evaluated further by categorising ETT into different groups to determine the overall trend of the results. Adjusted odds ratios showed a significant fall in chances of pregnancy across all ETT groups (Table 4.1.3.1-2). There was also evidence of clinical pregnancy changes but they only reached significance in some of the ETT groups (Table 4.1.3.1-3).

(a) Pregnancy rates

When pregnancy rates were assessed in the 6-ETT group model a sharp fall was particularly evident as the ETT extended beyond 120 seconds (Figure 4.1.3-1). The mean pregnancy rate for all ET procedures lasting 90-119 seconds were 75.7% but fell to 62.0% when ETT increased from 120 seconds to over 150 seconds. Pregnancy rates then appear to plateau in this study group and remain between 60-65% as ETT increases further (Table 4.1.3.1-2). The odds that a grade A or B blastocyst will implant successfully falls by 43% in the 2-ETT group model as ETT increased to over 120 seconds (p = 0.001).

(b) Clinical pregnancy rates

When clinical pregnancy outcomes were evaluated in the 6-ETT group model the falling adjusted odds ratio was not significant (p=0.145). The Hosmer lemeshow 'goodness of fit' was 0.219 suggesting a poor fit between the 6-ETT group model and the data. Studying the data graphically a pattern was apparent and so further analysis was undertaken (Figure 4.1.3-2). Categorising ETT into the 3-ETT group and 2-ETT group models (both contain tighter groupings around the same category of '< 120 seconds') resulted in a highly significant fall in the odds of a clinical pregnancy occurring (Table 4.1.3.1-3). In the 2-ETT group model the odds of a clinical pregnancy occurring fell by 34% as ETT increased over 120 seconds (p = 0.011) and in the 3-ETT model the odds fell by 31% as ETT increased to more than 120 seconds (p = 0.035). The Hosmer lemeshow 'goodness of fit' was 0.675 and 0.646 respectively for these models suggesting a much better fit with the data.

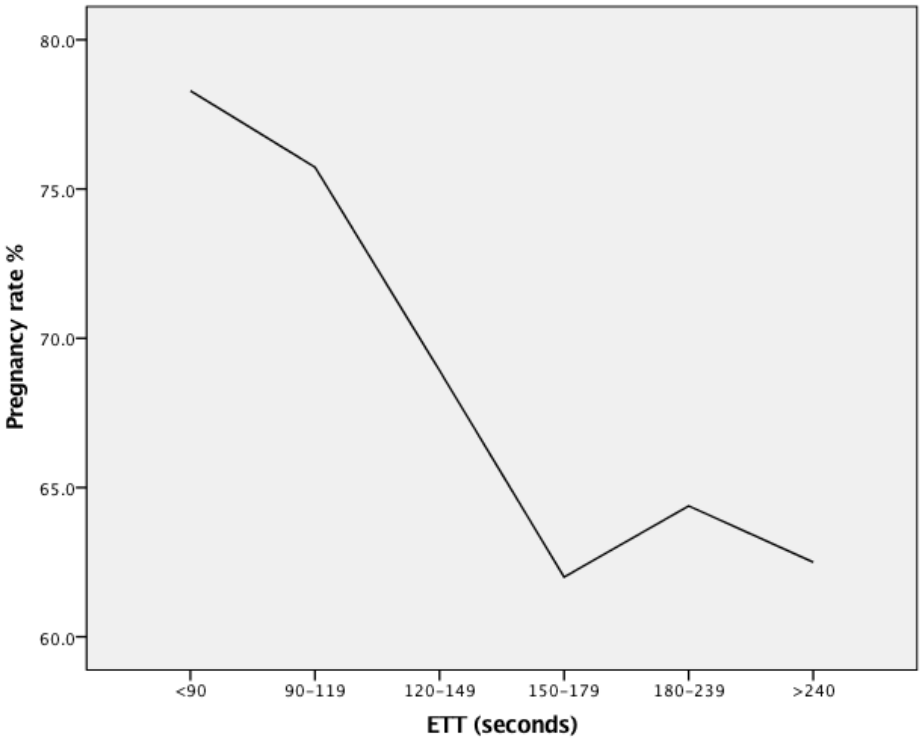


Figure 4.1.3-1 Pregnancy rates for all embryos according to ETT groupings

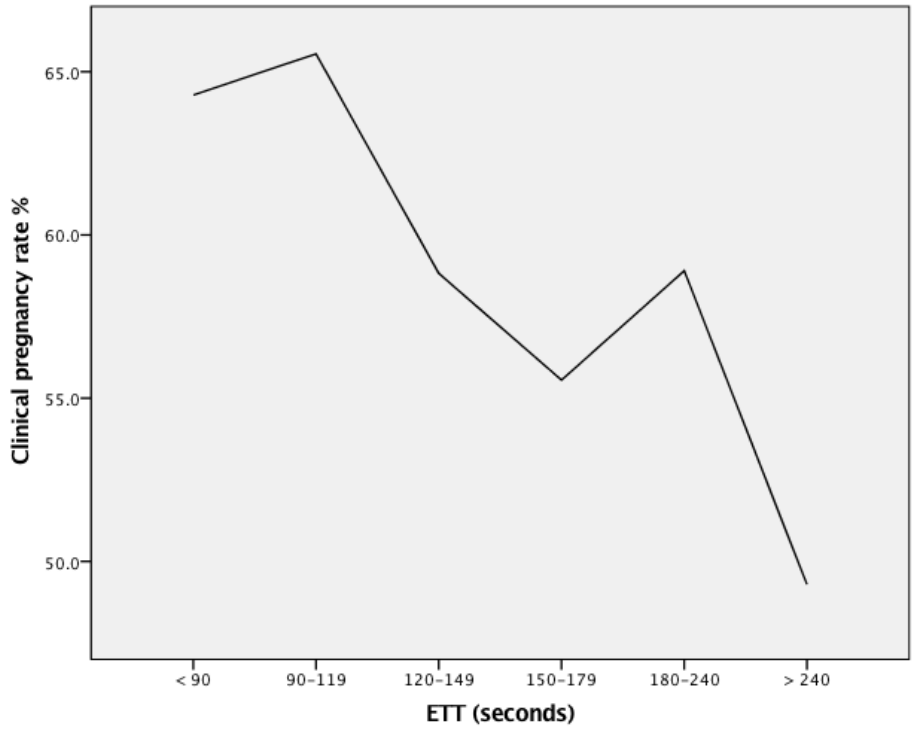


Figure 4.1.3-2 Clinical pregnancy rates for all embryos according to ETT grouping

Table 4.1.3.1-2 Multivariate analysis of pregnancy rates for all embryos based on ETT groupings and embryo grade with odds ratios and 95% CI

6 ETT group model	**n =**	**Pregnancy (%)**	**OR**	**± 95% CI**	**OR (adj)**	**± 95% CI**
<90	154	77.9	1		1	
90-119	238	75.6	0.88	0.54-1.42	0.78	0.47-1.31
120-149	153	69.3	0.64	0.38-1.07	0.58	0.34-1.00
150-179	45	60.0	0.43	0.21-0.86	0.35	0.17-0.74
180-239	73	64.4	0.51	0.28-0.94	0.47	0.25-0.89
>240	71	63.4	0.49	0.27-0.91	0.43	0.22-0.84
P-value			*0.031*		*0.017*	
3 ETT group model						
<120	392	76.5	1		1	
120-179	198	67.2	0.63	0.43-0.92	0.60	0.40-0.89
>180	144	62.9	0.54	0.36-0.82	0.53	0.34-0.81
P-value			*0.005*		*0.004*	
2 ETT group model						
< 120	392	76.5	1		1	
>120	342	65.8	0.59	0.43-0.82	0.57	0.40-0.79
P-value			*0.001*		*0.001*	
Continuous ETT model						
	734		0.999	0.99-1.00	0.999	0.99-1.00
P-value			0.067		0.045	
Embryo grade						
Grade A	431	74.5	1		1	
Grade B	303	67.3	0.71	0.51-0.98	0.69	0.48-0.99
P-value			*P = 0.035*		*P = 0.044*	

Table 4.1.3.1-3 Multivariate analysis of 'clinical pregnancy' rates for all embryos based on ETT groupings and embryo grade with odds ratios and 95% CI

6 ETT group model	n =	Clinical pregnancy (%)	OR	± 95% CI	OR (adj)	± 95% CI
<90	154	64.3	1		1	
90-119	238	65.5	1.06	0.69-1.62	0.96	0.61-1.50
120-149	153	58.8	0.79	0.50-1.26	0.70	0.43-1.14
150-179	45	55.6	0.69	0.35-1.36	0.58	0.29-1.18
180-239	73	58.9	0.80	0.45-1.41	0.74	0.41-1.35
>240	71	49.3	0.54	0.31-0.96	0.50	0.27-0.92
P-value			*0.170*		*0.145*	
3 ETT group model						
<120	392	65.1	1		1	
120-179	198	58.1	0.74	0.52-1.06	0.69	0.48-1.00
>180	144	54.2	0.63	0.43-0.94	0.63	0.42-0.95
P-value			*0.045*		*0.035*	
2 ETT group model						
< 120	392	65.1	1		1	
>120	342	56.4	0.70	0.52-0.94	0.66	0.49-0.91
P-value			*0.017*		*0.011*	
Continuous ETT model						
	734		0.999	0.99-1.00	0.999	0.99-1.00
P-value			0.061		0.057	
Embryo grade						
Grade A	431	64.3	1		1	
Grade B	303	56.4	0.72	0.53-0.97	0.66	0.47-0.93
P-value			*0.032*		*0.017*	

4.1.3.2 Based on embryo grade

There were no significant differences in patient or cycle characteristics between A and B blastocyst groups, except for the number of blastocysts available for transfer. Patients having a single high quality (Grade A) blastocyst transferred were likely to have a significantly higher number of blastocysts available for transfer. The mean number of blastocysts available for transfer were 6.0 (± 3.3) and 3.6 (± 5) respectively for Grade A and B embryo groups (Table 4.1.3.1-1).

As the quality of the embryo dropped from a grade A to a grade B blastocyst there was a fall in pregnancy and clinical pregnancy rates by 7.2% and 8.3% respectively. The odds (adjusted) of pregnancy and clinical pregnancy fell by 31% (OR 0.69, ± 0.48-0.99) and 34% (OR 0.66, ± 0.47-0.93) respectively (Table 4.1.3.1-2 and Table 4.1.3.1-3) when a grade B embryo was transferred instead of a grade A blastocyst. This fall was highly significant and supports the fact that embryo quality has a significant impact on pregnancy rates (p = 0.044) and clinical pregnancy rates (P = 0.017). The pregnancy and clinical pregnancy rates when separated by embryo grade fell across the six ETT groups but did not reach statistical significance (Table 4.1.3.2-1). A Cochran-Armitage 'test of trend' (P <0.05) was calculated to assess for an association between ETT and pregnancy rates. Since there appeared to be an association between the ETT and pregnancy and clinical pregnancy rates it seemed reasonable to re-analyse this data according to embryo grade in a logistic regression model adjusting for all the possible confounders as was done collectively for all embryos.

Table 4.1.3.2-1 Impact of ETT on pregnancy and clinical pregnancy rates in the context of blastocyst quality (Grade A or B)

Grade A	n =	Pregnancy %	Clinical pregnancy %
<90	98	76.5	64.3
90-119	137	79.6	70.1
120-149	86	73.3	65.1
150-179	30	66.7	63.3
180-239	40	67.5	65.0
>240	40	67.5	55.0
P-value		*0.397*	*0.646*
P-trend		*0.054*	*0.255*
Grade B	n =	Pregnancy %	Clinical pregnancy %
<90	54	81.5	64.8
90-119	102	70.6	62.7
120-149	62	62.9	51.6
150-179	20	55.0	55.0
180-239	33	60.6	51.5
>240	32	56.3	43.8
P-value		*0.081*	*0.289*
P-trend		*0.005*	*0.023*

4.1.3.3 Interaction of ETT and embryo quality

When ETT and embryo grade were examined jointly in a multiple regression model adjusting for confounders, there was shown to be no significant interaction between ETT and embryo quality, hence excluding any interaction that might affect pregnancy and clinical pregnancy rates (P = 0.851 and 0.946) and this is also shown graphically in Figure 4.1.3-3 and Figure 4.1.3-4 as the falling rates appear to follow a similar trajectory. Therefore, it is reasonable to report that the fall in pregnancy rates and clinical pregnancy rates appears to be affected by ETT but this effect appears to be independent of the quality of that embryo.

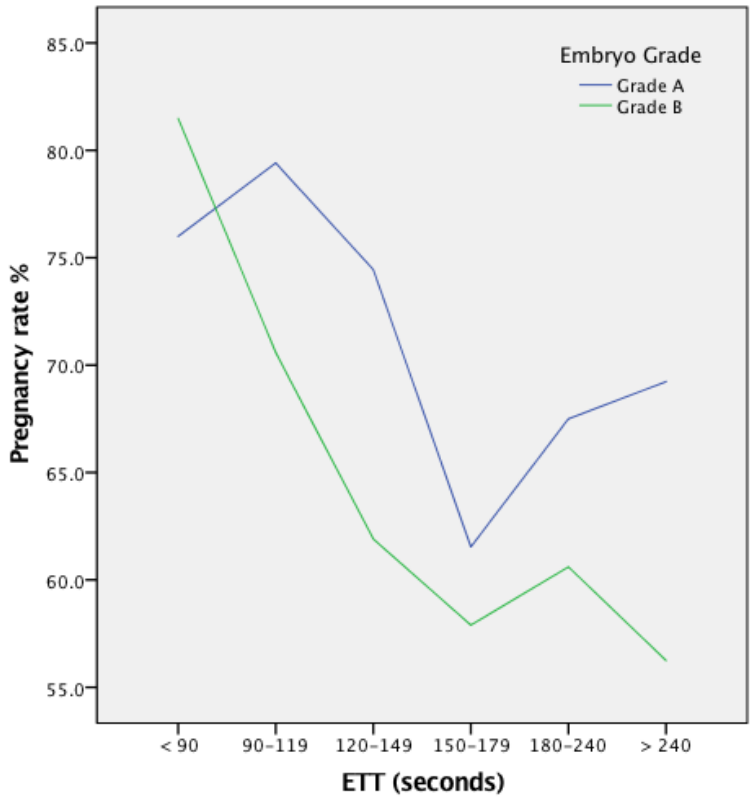


Figure 4.1.3-3 The effect of increasing ETT (seconds) on pregnancy rates according to Embryo grade (A/B), based on ETT categorised into six groupings

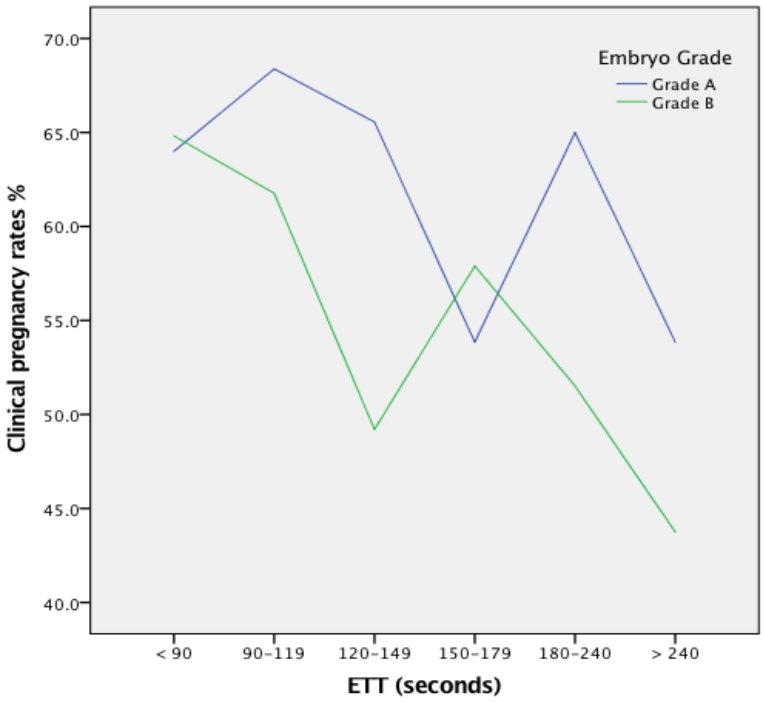


Figure 4.1.3-4 The effect of increasing ETT (seconds) on clinical pregnancy rates according to Embryo grade (A/B), based on ETT categorised into six groupings

4.1.4 Discussion

This is the first study in the literature, to consider single blastocyst cycles and examine the effect of ETT in the context of embryo quality. The principal finding of this study is that a multivariate analysis has found that increasing ETT significantly decreases pregnancy and clinical pregnancy rates is ($p = 0.045$ and $p = 0.057$), in agreement with the original work by Matorras et al. (2004). Ciray et al (2007) suggested that good quality embryos were not affected by prolonged ETT and that perhaps only poor quality embryos are affected, though the small numbers of poor quality embryos made it difficult to draw meaningful conclusions. This study has shown that increasing ETT appears to be associated with a significant downwards trend ($P_{trend} < 0.05$) in pregnancy rates and possibly also clinical pregnancy rates for both grade A and B embryos, independent of their quality. The multivariate analysis controlling for embryo quality has shown that there was no significant interaction between embryo grade and ETT affecting these implanting blastocysts.

In this study, ET 'difficulty' was not considered because it had already been identified in Chapter 2 that the current grading scales are not fit for purpose. This study therefore focused on ETT as a potential marker of ET 'difficulty' but also recorded surrogate markers (blood on the catheter and use of a tenaculum) associated with the more difficult ET procedures. As ETT increases it is suggested from this data there is a negative impact on pregnancy and clinical pregnancy rates that is independent of embryo quality. Understanding the mechanism responsible for this fall in prognostic potential of the embryo will require further work.

Mattoras et al. (2004) studied cleavage stage embryo transfer of multiple embryos but suggested that the fall in success rates were significant after 120 seconds. In this study of blastocyst stage embryo transfer both pregnancy and clinical pregnancy appears to be affected significantly once ET procedures are prolonged over 120 seconds and this is the case for embryos of differing quality. It is difficult to be certain about the accuracy of the '120-second' window reported by Mattoras et al. (2004) as that study did not detail exactly how they had recorded the ETT. It is assumed that the ETT published in that study were precise and represent the time the embryo was in the catheter until successfully deposited in the uterus.

4.1.4.1 Limitations

The ETT has been recorded in this piece of work by the embryologist loading the ET catheter. It reflects the time from a successfully loaded ET catheter until it has been checked to ensure it is empty at the end of the procedure. There is slight bias here to be considered as the ETT has been recorded by the embryologist and their own skill and speed may affect how long it takes them to check the catheter. The loading time was measured but the time to check was not. It was decided to include the time taken for the embryologist to check the catheter in the total ETT, as embryos may be retained on occasions. The embryologists may vary by a few seconds in the length of time they take to check the catheter but this was felt to introduce only minimal bias. It was also noted that patients having poorer quality embryos replaced had significantly fewer embryos to choose from and this itself therefore must be

considered as a source of bias This was controlled for in the multivariate analysis and but did not seem to be an important confounder in the analysis.

4.1.5 Conclusions

This chapter has highlighted the importance of ETT on the outcome of IVF cycles. If an embryo is exposed for a prolonged time during ET it is likely that this will significantly impact on its potential to implant and form a clinical pregnancy. Whilst this important effect has been reported here for the first time in single ET cycles it is now known for the first time that there is no interaction between ETT and the quality of the embryo. The next stage of this research is to look at the ET process in more detail and try to understand whether anything is happening to the embryo or the culture media that may affect the cells of the embryo. It is clear from this study that a blastocysts potential to implant is significantly reduced when the ETT exceeds '120-seconds'. If ETT is to become part of a clinically useful future grading system of ET 'difficulty' an attempt must be made to understand the biological plausibility of this effect, and this will be considered in the next chapter of this thesis.

Chapter 5

Impact of time exposure on gamete and embryo potential

5.1 THE IMPACT OF TIME EXPOSURE ON CULTURE MEDIA

5.1.1 Introduction

The ET procedure is a simple technique involving several steps to replace the embryo in the uterus. It has been debated by previous authors whether the 'embryo transfer time' (ETT) is an important factor that influences IVF success rates (Ciray et al., 2007, Matorras et al., 2004). In 'chapter 4' the data suggested that the ETT is an independent factor that affects a single blastocysts potential to implant independent of that embryo's quality. The time taken to transfer the embryo, despite the use of different techniques, is a continuous variable that applies to every ET procedure, yet little is known about the underlying mechanisms that affect the embryos.

In the laboratory at Nurture Fertility the embryos are cultured in the dark in large incubators (ThermoFisher) where the environment is clean and a steady environment of 5% carbon dioxide (CO_2), 37 degrees centigrade (°C) and 100% humidity maintained. The embryos develop in culture right up to the point of ET when they are moved into pre-prepared dishes containing G2 Plus culture media, just before the ET procedure itself, and maintained within the smaller MINC desktop incubators (K-MINC-1000, Cook Medical, US).

The dishes used during ET are prepared the day before and stored in the tightly controlled conditions inside the large incubators (ThermoFisher) for several hours. Each dish has been loaded with 1 millilitre of G2 Plus culture media (Vitrolife,

Sweden) which is a bicarbonate buffered medium containing hyaluronan and human serum albumin. The media in the ET dish is left to equilibrate in the incubator overnight. A further 1 millilitre of G2 Plus culture media is prepared and stored alongside the standard ET dishes, and this is used for flushing and loading the ET catheter (SureView Wallace Embryo Replacement Catheter CE118; Smiths Medical. On the day of ET, the embryos are prepared for transfer immediately prior to the procedure. Embryos that have been selected for transfer are removed from cell culture and placed into the pre-prepared ET dish and stored in the MINC desktop incubator.

At the point, the clinician is ready to attempt to pass the ET catheter loaded with the embryo through the cervix the ET dish is removed from the MINC desktop incubator. At this point the embryologist loads the catheter with the G2 Plus media and the embryo is loaded between two air bubbles in the tip of the catheter in 20μl of G2 Plus media. Once this is completed the ET catheter is transferred to the theatre for the clinician to undertake embryo transfer. Each ET dish has a lid that is kept closed during the ET procedure except for when the embryo is being loaded and the dish rests on a warmed plate in the flow hood to help maintain 37 °C.

During the ET procedure once embryos are removed from the incubator they are exposed to the normal environment, where the composition of regular air is 78% nitrogen (N_2) and 21% oxygen (O_2). The carbon dioxide content of room air is less than 0.1%, which means the bicarbonate buffered G2 Plus media that is loaded into both the ET dish and ET catheter is exposed to a far less controlled environment than

exists within either the larger or MINC desktop incubators. A few studies have demonstrated the detrimental effects that environmental factors can have on oocytes and embryos such as light and temperature (Cohen et al., 1997, Evans et al., 1999, Rocha et al., 1998). In trying to explain what is happening to the embryo as ET procedures are prolonged, this experiment set out to mimic exactly what is occurring in the laboratory and measure how much these parameters change.

5.1.2 Methods

To test the changes in pH, temperature and osmolality of the culture media in the ET dishes and Wallace SureView ET catheters occurring at the time of ET, a series of short experiments were undertaken. Twenty-four hours before the experiments started six ET dishes and six 2ml test tubes were prepared each containing 1 millilitre of G2 Plus media. Once filled the dishes were covered with a lid and the test tubes containing the culture media were stored in the large incubators in conditions where a stable environment of 5% carbon dioxide (CO_2), 37°C and 100% humidity was maintained. Additionally, ET catheters were warmed and stored in the incubator overnight.

5.1.2.1 Culture media pH

Prior to commencing the experiment the pH probe (Metler Toldeo, UK) was cleaned and calibrated. Cleaning of the pH probe with distilled water was performed at the start and end of each measurement taken. Calibration checks of the pH meter probe

were undertaken using two solutions of fixed pH; (1) 7.01 and (2) 4.02, and recorded to be accurate at the start and end of experimentation. The pH was recorded in both ET dishes and catheters.

(a) Culture dishes

A single ET dish was removed from the incubator and placed on a standard warmed plate (37°C) in the ET hood. The lid was kept in place until a control sample of 20μl of culture media was removed from the well with a graduated pipette. The timer was started and the pH of this sample was immediately recorded, allowing 10 seconds for sampling and reading of the correct value. A new pH measurement was taken at baseline (control), 1, 2, 3, 4 and 5-minute intervals with every 20μl sample of G2 culture media that was removed from the dish, ensuring the lid was replaced on the dish between sampling. Time intervals were chosen to reflect a reasonable exposure time that might be expected to occur during most easy and difficult ET procedures. In between each measurement, the pH probe was cleaned with distilled water. This was repeated for three separate ET dishes.

(b) ET catheter

Additionally, the change in pH was measured in the tip of the Wallace SureView catheter over the same intervals. A warmed SureView Wallace ET catheter and 1ml syringe was removed from the incubator with the mini test tube containing 2mls of G2 media. One millilitre of G2 media solution was immediately loaded into the catheter as would be performed during a normal ET procedure, forming a continuous column of media through the catheter lumen and into the syringe. A

small volume equivalent to about 20µl was ejected and pH tested at intervals to measure how this changed over time in the tip of the catheter. The catheter in-between measurements rested on the warmed plate in the hood. The pH probe was similarly cleaned and calibrated in between measurements and at the beginning of experimentation.

5.1.2.2 Culture media temperature

Two media dishes containing 1ml of G2 media were removed from the large incubators, where they had equilibrated overnight, and they were transferred to the MINC desktop incubator. Separately each dish was then removed from the MINC incubator and placed on a warmed plate as would happen during a real ET procedure. The lid was removed immediately so that a temperature probe (thermometer 4600 with YSI probe) could measure the temperature of the culture media as the timer started. The same procedure was performed at 30-second intervals replacing the lid between measurements to mimic the same conditions that occur during ET. The lid of the dish would probably have been off the dish for longer during embryo loading but this seemed the closest way to accurately measure the fall in temperature in the dish exposed to the environment of the ET hood. Room temperature was recorded at the start and end of each experimental run.

5.1.2.3 Culture media osmolality

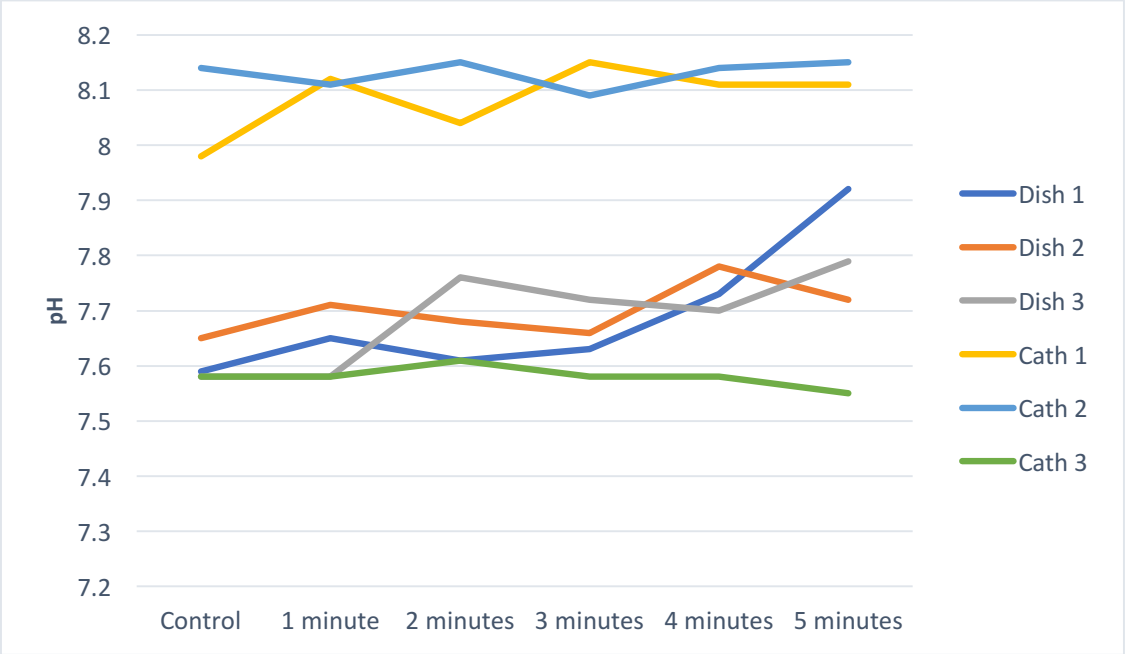
The osmolality of the culture media was tested in both the ET dish and the ET catheter at the start and end of 5 minutes to see if there was a significant change during this time. The digital osmometer (Vitech Scientific, USA) took two minutes to complete a measurement and so taking a series of osmolality readings time intervals was only deemed necessary if a significant change in osmolality was seen. The extreme of 5-minutes was tested initially and if a significant change noted then further measurements at 2, 3, and 4-minute intervals would be taken using separate ET dishes. The limitation of the digital osmometer was the time to took to read a sample, and this prevented paired readings being taken from the same dish consecutively as had been done with pH and temperature.

5.1.3 Results

The pH of the G2 culture media in both the ET dishes and ET catheter tip did not change significantly over the 5-minute period that was tested. The media pH remained stable over this time with no evidence of significant fluctuation from the control value. The media loaded into catheter 1 and 2 started at a higher control pH value of 7.98 and 8.14 respectively. This higher starting pH values seen in the catheter series may have been due to a problem with the G2 culture media equilibrating overnight in screw top test tubes. Whilst the starting values were initially higher in these two samples there was still no evidence of a change in pH over the 5-minute period (Figure 5.1.3-1). Calibration of the pH probe was correct at

the start and end of the experiment with values falling within 0.1 of expected control ph.

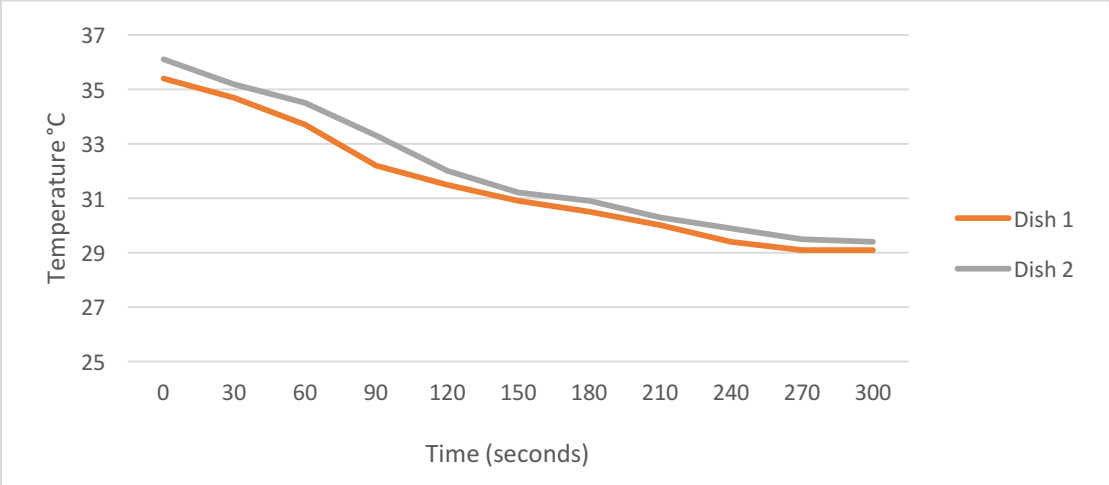
Figure 5.1.3-1 Effect of time on pH of culture media in ET dishes and ET catheter tip exposed to normal environment



Room temperature was 24.0 °C during both the pH and temperature experiments. The temperature of the MINC incubator was recorded as 36.8 °C and once the dishes were removed the temperature of the culture media fell significantly over the 5-minute period. The fall in temperature was not linearly related to time but there was a fall of 6.3 and 6.7 °C in dish 1 and dish 2 respectively (Figure 5.1.3-2). The fall in temperature of a 20µl drop of G2 media in the tip of the Wallace SureView catheter was not tested, as there was no accurate way to do this, though it is reasonable to

assume that such a small volume to surface area exposed to room temperature would probably fall to room temperature within several seconds.

Figure 5.1.3-2 Temperature change measured in ET dishes over 5-minute period



There was no noticeable change seen in osmolality of the culture media in either the ET dish or ET catheter tip. The osmolality of the G2 media was recorded as 295 on removing it from the incubator and testing it immediately, and then after 5-minutes exposure to the environment the osmolality of the G2 media in the dish was 296 and in the tip of the catheter it was 298.

5.1.4 Discussion

This series of experiments have revealed that the most important factor that alters during the ET process is the temperature of the G2 media in which the embryos are transferred. The temperature falls by about 6.5 °C in the ET dishes containing 1ml of media over a 5-minute period, and it is highly likely therefore that the temperature

of the 20μl of G2 media in the tip of the ET catheter also falls significantly. An embryo cushioned in the tip of the ET catheter in this media would therefore be susceptible to significant cooling during transfer.

In 'chapter 4' it was shown that there is a significant fall of pregnancy rates with prolonged ET procedures and that ETT is an independent factor affecting IVF success rates. The embryo that is transferred will be exposed in the tip of the catheter to a significant fall in temperature and it is remains to be determined whether this cooling effect has a detrimental impact on the embryo's integrity and potential to implant. The non-buffered G2 culture media is exposed to normal room air rather than the tightly regulated CO_2 rich humidified incubator environment. However, it is of interest and importance that the pH and osmolality of the transfer media does not change significantly over a 5-minute exposure period.

An embryo unloaded from the ET catheter will no doubt have been exposed to these less than ideal conditions in the tip of the catheter during a difficult ET procedure. It is important to consider that the G2 media in the dish which the embryologist may unload this embryo may have cooled significantly. The most likely scenario in very difficult ET procedures is that the dish containing the embryo will then be returned to the MINC desktop incubator where CO_2 and temperature are optimal, while the clinician negotiates a successful path through the cervix. A reloaded embryo deposited in the uterus with a further attempt in these situations will have been exposed to swings of temperature and light exposure. The impact that the changes in the environment have on the implanting embryo needs further investigation.

5.1.5 Conclusions

During a prolonged ET, the embryo may be exposed to significant cooling of the transfer media both in the ET catheter but also in the ET dish if at any point the embryo is unloaded. The next series of experiments will look closely at this phenomenon to decide whether this affects the development and prognostic potential of the embryo to implant.

5.2 THE IMPACT OF TIME EXPOSURE ON THE MOUSE EMBRYO

5.2.1 Introduction

It is known that a prolonged ET procedure has been associated with reducing an embryo's potential to implant (Matorras et al., 2004). This theory was then rejected by Ciray et al (2007), who claimed no change was seen in implantation rates of good quality embryos following a prolonged exposure time (Ciray et al., 2007). Both studies assessed cleavage stage embryos at embryo transfer. Many clinics around the world perform cleavage stage embryo transfers, but as culture techniques improve there are many more blastocyst stage ET procedures taking place. A Cochrane review showed that there is moderately good evidence to suggest clinical pregnancy rates are higher with fresh blastocyst ET (Glujovsky et al., 2016).

It has been shown for the first time in chapter 4 that increasing ETT can have a detrimental impact on the prognostic potential of a single blastocyst to implant. In agreement with results from Matorras et al. (2004), it would appear that > '120-seconds' exposure time seems to be critical in terms of seeing a significant fall in implantation rates. This data indicated that the effect of increasing ETT on implantation rates is independent of embryo quality, and that all embryos are affected to the same degree.

This research has focused entirely on the human blastocyst to this point and the impact of ET 'difficulty' on its prognostic potential. Increasing ETT appears to be an

important factor, but there is still no deeper understanding of the mechanism driving the lower implantation rates. It seems plausible based on the results in chapter 5.1 that the significant temperature drop as ETT increases may be critical, and it must be considered whether the cooling of the culture media is affecting the embryo's potential to implant.

The next stage of this research is to study the effect of time exposure on the development of the mouse embryo using morphokinetics. It is logical to look at the mouse embryo's cellular development and try to find an explanation for the effects seen in vivo in the human. This pilot data was undertaken in the hope that by studying mouse embryo development may explain why prolonged ETT seems to affect an embryos potential to implant.

5.2.1.1 Morphokinetics

Techniques to monitor embryo development in vitro have improved significantly over the last 25 years. The potential of an embryo to implant has been associated with morphokinetic changes captured with time lapse imagery mostly during the cleavage stages (Meseguer et al., 2011). Time-lapse imagery is a technique that follows the developing embryo in culture and captures an image of the cleaving embryo at intervals. The time-lapse images make it possible to observe whether an embryo reaches key development stages appropriately (Figure 5.2.1-1). It is the time taken to reach these key embryo development stages and the expansion area of the blastocyst that are important.

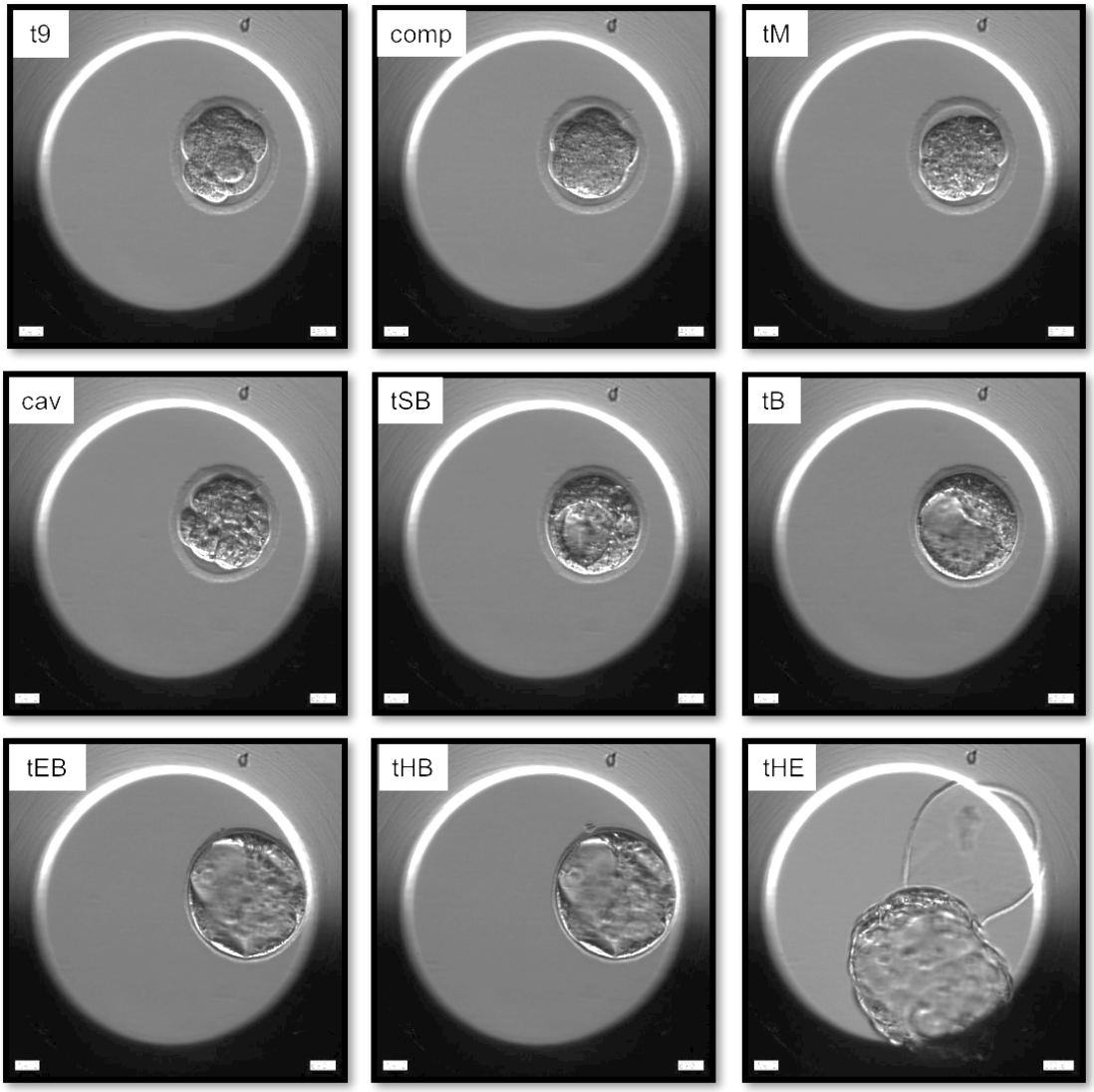


Figure 5.2.1-1 Embryoscope time-lapse evaluation of cleavage embryo development to hatching blastocyst illustrating key morphokinetic stages: t9 - time from thawing to the formation of a nine-cell embryo, comp - time from thawing to compaction of embryo, tM - time from thawing to formation of a morula, cav - time from thawing to formation of cavity, tSB - time from thawing to 'start of blastulation, tB - time from thawing to formation of full blastocyst, tEB - time from thawing to hatching blastocyst, tHB - time from thawing to a completely hatched embryo (See table 16)

Time lapse imaging allows different morphokinetic characteristics of the developing embryo to be followed, and several studies have shown these may be correlated with subsequent implantation success. It has been shown that the time of morula formation (tM) predicts blastocyst formation rates and the time for expansion of blastocyst (tEB) predicts implantation (Motato et al., 2016). Therefore, if a prolonged ETT exposure affects an embryo's potential to implant it seems plausible that it may be possible to see morphokinetic changes in the embryo. In addition, a lower total cell count (TCC) of the pre-implantation embryo has been associated with reduced viability and potential to implant (Papaioannou et al., 1989, Lawitts and Graves, 1988, Tsunoda and McLaren, 1983).

There have been no studies in the literature identified that have looked at the effect of the cooling of culture media on the morphokinetic changes in the developing embryo. It seemed logical first to study the impact of prolonged ETT in mouse embryos and obtain some pilot data to see if an effect was evident, before considering the human embryo, which inevitably would have involved more complex ethical approval to examine in this way. The mouse embryo is an ideal place to test the hypothesis that exposure of embryos to cooling with a prolonged ETT may affect morphokinetic development of the embryo and implantation potential.

5.2.1.2 Mouse embryo epigenetics

The mouse embryo is different to the human embryo in its development and so the results here must be considered in context. The mouse genome is activated far

earlier on day-1 of cleavage compared to the human embryo, whose genome activates later around the 8-cell stage (Wang et al., 2004, Dobson et al., 2004). The mouse embryo develops at a different rate to the human reaching compaction and blastocyst at different times to the human embryo, and has one further level of cell division at blastocyst (Niakan et al., 2012). The genome of the developing mouse embryo begins to influence cellular development earlier than in the human, but it remains a simple way to test whether prolonged ETT and embryo exposure affects morphokinetic parameters.

This set of experiments was designed to study the effects of a prolonged ETT on the embryo by analysing how increasing time exposure in a simulated mouse embryo model affects development. The effects of a prolonged ETT was studied at two different stages of embryo development:

- Experiment 1 - cleavage stage embryos
- Experiment 2 - blastocyst stage embryos

5.2.2 Methods

The morphokinetic variables of embryo development were assessed with time lapse photography following different amounts of stress simulating a prolonged ETT. The stress of a prolonged ET procedure was simulated by exposing mouse embryos in an ET catheter for different periods of time. This laboratory based experiment was undertaken at the University of Nottingham.

5.2.2.1 Embryo culture

The B6C3-F1 and B6D2 F1 mouse embryos were purchased frozen at the one cell stage (Embryotech Laboratories, Inc., Massachusetts, USA) and were thawed in G-IVF culture media (Vitrolife, Sweden) according to supplier's instructions (Appendix 1). After thawing, the embryos they were cultured in G-1 media (Vitrolife, Sweden) for experiment 1 and in GTL medium (Vitrolife, Sweden) for experiment 2 under mineral oil (Vitrolife, Sweden) in incubators with humidified conditions at 37°C and 5% CO_2.

5.2.2.2 Embryo stress and onward culture

Embryos were removed from the incubator and placed into a pre-warmed 20 µl G-2 media drop (Vitrolife, Sweden) and then loaded into a pre-warmed 18-centimetre soft embryo replacement catheter (SureView Wallace embryo replacement catheter CE118; Smiths Medical) between two air bubbles to simulate human ET (Figure 5.2.2-1).

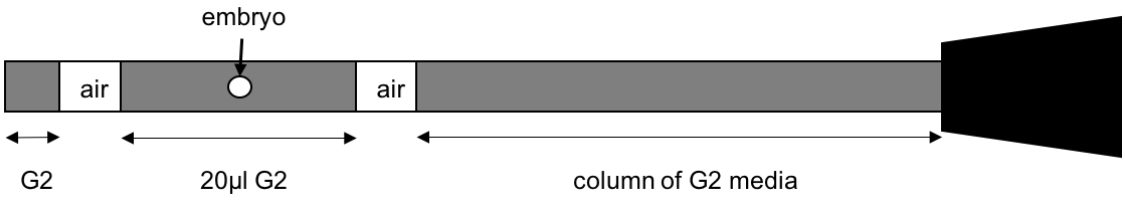


Figure 5.2.2-1 Diagrammatic of ET catheter loading procedure

(a) Experiment 1

During experiment 1 the embryos were stressed around the 8-cell stage (41-42 h post-thawing) The embryos were separated into 3 groups: (a) control group without manipulation; (b) 2-3 min group, where embryos were left in the catheter at room temperature (RT) for 2-3 minutes mimicking a clinically easy ET and (c) 7-8 min group, where embryos were left in the catheter for 7-8 minutes at RT mimicking a clinically very difficult embryo transfer.

(b) Experiment 2

During experiment 2 embryos were stressed at blastocyst stage (77-78 h post-thawing). The embryos were separated into 3 groups: (a) control group without manipulation; (b) 2-3-min group, where embryos were left in the catheter at room temperature (RT) for 2-3 minutes mimicking a clinically easy embryo transfer and (c) 4-5 min group, where embryos were left in the catheter for 4-5 minutes at RT mimicking a clinically difficult embryo transfer.

5.2.2.3 Time-lapse imaging

After embryo stress, the embryos were cultured individually in G-2 media (Vitrolife, Sweden) in a time-lapse incubator (EmbryoScope, Unisense FertiliTech, Denmark) at 37°C and 5% CO_2 where embryo pictures were taken every 15 min in 10 different focal planes. Embryo images were analysed by EmbryoViewer image analysis software.

The following morphokinetic criteria was noted (Table 5.2.2.3-2): the embryo size immediately after embryo stress, 24 hours after stress and at the start of embryo hatching; division time from thawing to 9-cells (t9); time to form a morula (tM); start time of embryo cavitation (tSB); time to blastocyst stage, when the blastocoel occupies more than 50% of the embryo (tB); time to blastocyst expansion grade, when zona pellucida thins by more than 50% (tEB); time to start of embryo hatching (tHB) and time of full embryo hatching (tHE). The expansion grade of embryos reaching blastocyst was scored according to the morphological classification criteria outlined in Table 5.2.2.3-1 (Schoolcraft et al., 1999). It was noted if hatching occurred at multiple sites. These parameters were analysed for each embryo and collated for further analysis of the groups.

Table 5.2.2.3-1 Standard morphology classification criteria during day-5 embryo scoring (Gardner and Schoolcraft, 1999).

Blastocyst expansion grade	Interpretation
6	Hatched blastocyst: the blastocyst has completely broken out from the breach in the ZP naturally.
5	Hatching blastocyst: the blastocoele chamber/space is larger than the original volume of the embryo and the trophectoderm is herniated through the zona pellucida.
4	Expanded blastocyst: the blastocoele is greater in volume than the original volume of the embryo and the zona has started to thin/dilute/disperse.
3	Full blastocyst: the embryo is being filled completely by the blastocoel
2	Blastocysts: the blastocoele occupied half or more than half of the embryo volume
1	Early blastocyst: with blastocoele occupied less than half the volume of the embryo

Table 5.2.2.3-2 Definitions of morphokinetic variables used in time-lapse imaging evaluation

Parameter	***Definition***
t9	Time from thawing to the formation of a nine-cell embryo
comp	Time from thawing to compaction of embryo
tM	Time from thawing to formation of a morula, where more than or equal to 90% of cells were compacted and individual blastomeres were unidentifiable
cav	Time from thawing to formation of cavity
tSB	Time from thawing to 'start of blastulation', when the earliest signs of cavitation were distinctly visualised
tB	Time from thawing to formation of full blastocyst, when the blastocoel occupied more than or equal to 50% of the embryo
tEB	Time from thawing to expanded blastocyst, when the blastocoel fully occupied the embryo and stopped increasing in size, and the zona pellucida started to thin
tHB	Time from thawing to hatching blastocyst, when the trophectoderm herniation/extrusion were first observed through the zone pellucida
tHE	Time from thawing to a completely hatched embryo

5.2.2.4 Measuring the size of blastocysts

Blastocyst area (μm^2) as demarcated by the zona pelucida (ZP) was measured using the EmbryoViewer image analysis software immediately after stress, 24-hours after stress and at the start of hatching (Figure 5.2.2-2)

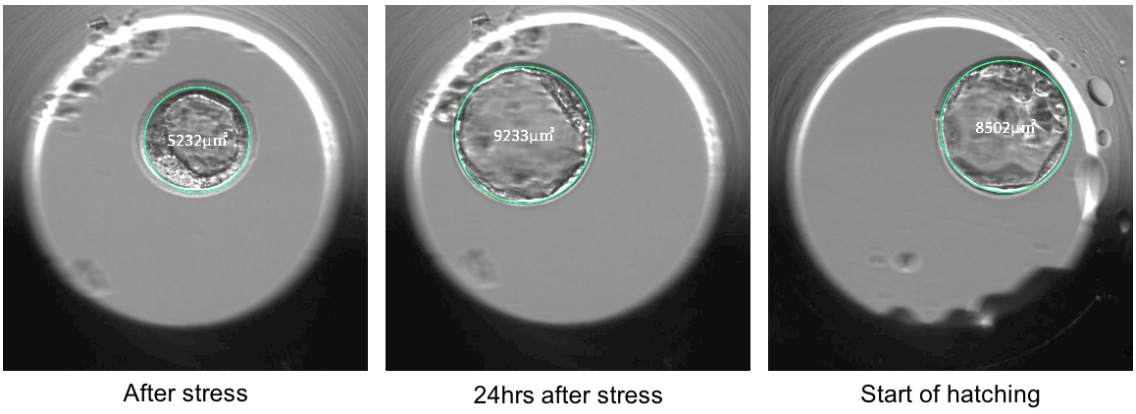


Figure 5.2.2-2 Blastocyst area (μm^2) measured in Embryoscope – green circle denotes the area of embryo measured inside the zona pellucida

5.2.2.5 Total cell counts at blastocyst stage

Total cell counts (TCC) were calculated manually at the end of experiment 2 with a nuclear staining technique using 4',6-diamidino-2-phenylindole (DAPI). DAPI is a fluorescent stain that is used in fluorescence microscopy to stain nuclei. The blastocysts were initially fixed in 4% paraformaldehyde (PFA)/ phosphate buffered saline (PBS) solution for 20 min at RT. The blastocysts were permeabilised in 0.1% Triton-100x/PBS for 2 minutes at RT. The embryos were then mounted on a superFrost microscope slide using Vectashield-DAPI as mounting medium and

nuclear staining. Total cell count was effectuated on a Nikon Eclipse Ti 90x microscope, along with a Hamamatsu digital camera (C4742-80-12AG) and a fluorescent filter corresponding to the excitation 350 nm and 470 nm of emission for DAPI. The DNA of all blastocyst cells fluoresced blue (Figure 5.2.2-3). Images were captured and analysed with Velocity 3D imaging software (PerkinElmer Inc.).

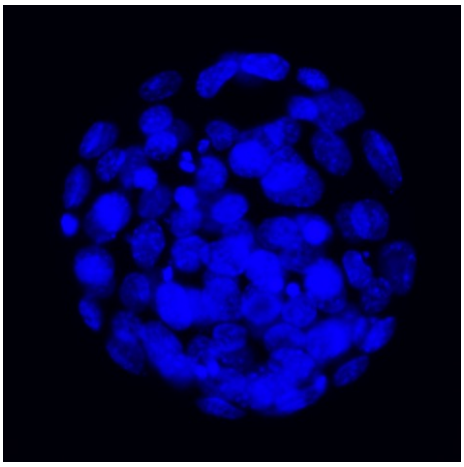


Figure 5.2.2-3 Fluorescent DAPI stain of mouse blastocyst for total cell count (TCC) as cell nuclei have been stained in blue.

5.2.2.6 Statistics

Statistical analysis was carried out with SPSS 23 (Statistical Package for Social Sciences; IBM, Chicago, IL, USA). All primary outcomes were continuous data. Normal distribution was checked by visual inspection of histograms and a student t-test or Mann-Whitney u-test if required. A one-way analysis of variance (ANOVA) was used to examine the significance of the differences between control and time exposure groups. A difference was statistically significant when $P<0.05$.

5.2.3 Results

5.2.3.1 Cleavage stage embryo stress

44 cleavage stage embryos at the 8-cell stage were divided between the control group and two further groups reflecting different levels of embryo stress. All embryos were stressed at the 8-cell stage. The most important observation with time-lapse imaging was that the time taken for the embryos to completely hatch (tHE) was prolonged in the 7-8-minute group. The tHE was 122.1 hours (±14.3) and 116.3 hours (±11.2) in the control and 2-3-minute groups respectively, but was prolonged at 135.8 hours (± 9.6) in the 7-8-minute group (p = 0.038). In addition to the prolonged tHE only 5 embryos out of 15 embryos completely hatched in the 7-8-minute group compared to the control and 2-3-minunte groups, where 8 out of 14 and 8 out of 15 embryos completely hatched respectively. No other morphokinetic parameters showed any significant differences across the groups (Table 5.2.3.3-2).

5.2.3.2 Blastocyst stage embryo stress

66 blastocyst mouse embryos were included in the second experiment and divided between the control and two further groups of embryo stress. All embryos were stressed at the blastocyst stage. In this series, the only morphokinetic parameter that significantly changed was that the time taken for the embryos to completely hatch (tHE) was shorter at 80.8 hours (± 7.4) in the 4-5-minute group (P = 0.025). In the control and 2-3-minute groups the time to complete hatching was 90.4 hours (±

14.9) and 87.9 hours (± 9.7) respectively. In the 4-5-minute group 26 out of 30 embryos completely hatched in a shorter time-period than the embryos in other groups. A total of 13 out of 14 embryos completely hatched in the 2-3-minute group but in the control group only 5 embryos out of 22 hatched completely. The embryos in the control group took on average 10 hours longer to hatch having not been exposed to any stress. No other morphokinetic parameters showed any significant differences (Table 5.2.3.3-2). There was no significant difference between the three groups when evaluating the number of embryos that had hatched at multiple sights on the ZP (p = 0.779). It does not appear that multiple hatching was relevant in this small sample of 66 embryos. In addition, there was no significant difference in the quality of the blastocysts assessed across the three groups, as determined by morphological expansion grade (p = 0.571).

5.2.3.3 Total Cell Count for embryos exposed to blastocyst stage stress

The TCC was calculated in 44 of the 66 embryos stressed at the blastocyst stage. The mean TCC was 131 cells (± 51) across all three groups but there was found to be no significant difference (p = 0.838) across the three groups (Table 5.2.3.3-1).

Table 5.2.3.3-1 Mean TCC, SD and SEM for three different groupings

Groups	**n =**	**Mean TCC**	**SD**	**SEM**
control	5	122	62	28
2-3 mins	13	137	45	12
4-5 mins	26	128	57	17
P-value		0.838		

Table 5.2.3.3-2 The impact of time exposure on cleavage and blastocyst stage embryos as assessed by morphokinetic changes in time-lapse incubation

Cleavage	**Control**				**2-3 mins**				**7-8 mins**				**p-value**
	n=	**mean**	**SD**	**SEM**	**n=**	**mean**	**SD**	**SEM**	**n=**	**mean**	**SD**	**SEM**	
t9	14	50.7	4.7	1.3	15	48.4	4.4	1.1	15	49.5	4.3	1.1	*0.388*
tcomp	14	42.7	1.94	0.5	15	42.1	0.6	1.6	15	43.2	1.9	0.5	*0.219*
tM	14	61.7	6.7	1.8	15	62.5	5.6	1.4	15	64.5	6.2	1.6	*0.458*
tcav	14	67.9	6.0	1.7	15	69.6	7.1	1.8	15	70.2	7.7	2.0	*0.661*
tSB	14	72.5	7.7	2.0	15	73.3	6.6	1.7	14	74.9	8.4	2.3	*0.694*
tB	14	77.0	8.6	2.3	15	78.5	8.5	2.2	14	78.7	9.9	2.6	*0.860*
tEB	13	84.3	10.7	3.0	15	91.1	13.2	3.4	12	90.3	17.0	4.9	*0.385*
tHB	14	91.3	22.9	6.1	15	88.1	16.2	4.2	14	85.2	19.7	5.3	*0.718*
tHE	8	122.1	14.3	5.1	8	116.3	11.2	4.0	5	135.8	9.6	4.3	*0.038*
ZP Area μm^2	14	8305	2110	564	14	8604	1243	332	13	8831	1796	498	*0.737*
Blastocyst	**Control**				**2-3 mins**				**4-5 mins**				**p-value**
	n=	**mean**	**SD**	**SEM**	**n=**	**mean**	**SD**	**SEM**	**n=**	**mean**	**SD**	**SEM**	
tB	18	79.9	11.4	2.7	7	83.1	3.5	1.3	7	78.5	3.6	1.4	*0.613*
tEB	18	85.0	13.1	3.1	13	87.8	11.9	3.3	21	80.7	6.1	1.3	*0.147*
tHB	17	95.4	20.8	5.0	4	107.7	10.7	5.3	8	99.5	17.1	6.0	*0.499*
tHE	5	90.4	14.9	6.6	13	87.9	9.7	2.7	26	80.8	7.4	1.4	*0.025*
ZP Area stress μm^2	22	6638	1598	341	14	6745	1420	380	30	6976	1227	224	*0.678*
ZP Area stress +24 μm^2	22	9280	1914	408	14	8954	1131	302	29	8724	1469	273	*0.463*
ZP Area hatch μm^2	5	8313	1266	566	6	8612	940	384	11	8239	1082	326	*0.795*

(1) Area measured in μm^2 just inside zona pellucida (2) Time measured in hours

5.2.4 Discussion

The main finding of this series of experiments relates to embryo hatching and that potentially a prolonged ET procedures may impact on an embryos potential to hatch and implant. However, the evidence is not conclusive and most of the morphokinetic parameters studied here did not change significantly when the embryos were exposed for longer periods of catheter stress. There are some important considerations though, as this study was initially designed with the intention of obtaining pilot data and a much larger study sample may well have revealed changes beyond what was seen here. Therefore, there are several limitations to this data interpretation but the data has given much to consider.

This research has focused on single blastocyst ET, and it is well known that time-lapse photography focuses on the cleavage stage embryo. However, if changes are seen in the morphokinetic parameters in a cleavage stage embryo it seems logical that this may be highly relevant for the more robust blastocyst containing many more cells. Embryos stressed at the cleavage (8-cell) stage appeared to take longer to hatch in the group exposed to catheter stress for 7-8 minutes. This group also had fewer embryos that completely hatched overall than in the other groups, suggesting an element of incomplete hatching taking place. Clinically the timing of ET is beneficial to implantation if blastocysts are transferred partially hatched or about to hatch (Chimote et al., 2013). Though it is known that transferring partially hatched embryos benefits implantation it may be a balance between exposing a fully hatched embryo and one that is partially hatched to potential cooling in the catheter during

'difficult' ET. Whether the effect of a prolonged ET time as simulated in this experiment affects the hatching potential of an embryo is unclear, but if it does it may have important implications for implantation. There is a possibility that prolonged exposure and cooling of cleavage stage embryos during ET may affect the outer ZP and its potential to hatch easily and consequent impact on implantation.

The embryos that were studied after catheter stress at the blastocyst stage also showed that the hatching potential differed across the three groups. The group of embryos exposed to cooling for 4-5 minutes appeared to hatch on average about 10 hours earlier than the control group. This difference was significant but the number of embryos that completely hatched in the control group was low (n =5) compared to the 2-3-min and 4-5 min groups where proportionally more embryos completely hatched overall. Catheter stress on the cleavage stage embryo and blastocyst must be considered separately as they relate to two different stages of embryo development. Although direct comparison is not possible it is of interest that the findings here are in complete contrast to what was found in experiment 1 when embryos had been stressed in the catheter at the cleavage cell stage. The explanation for this is unclear and in these small data sets difficult to ascertain with any accuracy. It is plausible that the developmental stage of the embryo is important when looking at the stress of prolonged ETT, as the cellular mechanisms for coping with this stress may differ in an embryo at different stages of development.

Blastocyst hatching rates may well be affected by prolonged exposure during 'difficult' ET but to explain this will require a much larger study sample. Earlier

hatching might be suggestive of zona pellucida weakening and thinning because of the catheter stress occurring at a crucial time when the expanding blastocysts internal fluid pressures are reliant on sodium-potassium osmotic changes. It is possible that cellular damage occurring at this crucial time might disrupt the embryo's delicate osmotic balance leading to higher pressures, swelling and earlier hatching but these results did not find any difference in overall blastocyst size at hatching so this theory may be incorrect.

The cellular apoptosis rates were not calculated in this second experiment but TCC in 44 hatched embryos was recorded. Although no significant difference was found to have occurred in TCC, based on the theory of cellular injury discussed here it may be useful in a future study to combine a TCC technique with TUNEL staining to assess apoptosis cell rates also to help determine the levels of embryo damage.

5.2.4.1 Limitations

The results here must be interpreted with caution in view of the small study sample. The study was limited by sample size as the main objective was to look at the effect of prolonged ET on the morphokinetic changes in a small number of embryos. Initial data collection had been designed to determine if there were any significant changes in the mouse embryo before repeating these experiments in an accurately powered human embryo sample with the necessary ethical approval. There is a disparity between the development of the mouse and human embryo and this is an important limitation of this data. Whilst it is known that the mouse embryo changes

morphologically in a similar way to the human embryo the mouse embryo differs to the human embryo in other ways. These are all factors that may hamper our ability to extrapolate our results and apply them directly to the human embryo.

5.2.5 Conclusions

It is plausible that an embryo's potential to hatch, as is seen here, is affected by media cooling during a prolonged ETT. However, this will require a further study and much larger sample of embryos to determine if this effect is real and because of the stress on the exposed embryos. There are simply too many variables in these mouse embryo experiments to be certain if the effect on hatching is due entirely to the catheter stress.

The optimal experiment would involve studying the effect of catheter stress on a larger sample of human blastocysts by investigating morphokinetic changes, TCC, and apoptosis rates. This next stage of experimentation was felt to be too complex and beyond the scope of this work at this stage. These results indicate that a further study may reveal changes occurring in the embryo during 'difficult' ET that could explain why prolonged ETT is important to implantation success. This next set of proposed experiments should help determine whether exposing blastocysts to the environment beyond the supposedly critical '120-seconds window' is reflected at a cellular level.

Chapter 6

Developing & Testing a new grading system

6.1 EXPT 1: THE RELIABIITY OF A NEW GRADING SYSTEM

6.1.1 Introduction

There is no nationally recognised grading system to assist clinician reporting of ET 'difficulty'. The results from 3.2.3 indicate that a carefully structured grading system that considers more than just the surrogate markers of difficulty such as; blood on the catheter or use of a tenaculum, may be beneficial. The much subtler markers of ET difficulty, relate to the delicate manoeuvres by the clinician to overcome resistance and successfully pass the catheter through the cervix. These were described by Kava-Braverman et al. (2017), who suggested that a clearly stratified grading system for these additional early manoeuvres is essential when studying the effect on IVF outcomes.

The systematic review results (Chapter 2) confirm there is currently a wide variation in how clinicians describe and record the ET procedure with little agreement across the world on how to define different levels of difficulty. It seems logical clinically and for future research purposes to simplify this system and draw clear boundaries of what are acceptable levels of difficulty. The use of a non-descriptive scale such as the VAS was examined in chapter 3.1 but has not resolved this issue, and so perhaps a well-tested grading scale, such as the graduated scale developed for use with the SureView Wallace catheter may prove a better solution. In the same way that embryo quality is graded it is important to have a robust way of consistently determining how difficult the ET procedure has been. The possibility of designing a

universally interchangeable ET ‘difficulty’ grading system that is functional in all clinics for all clinicians using all types of catheters has still to be determined.

The first stage of introducing a new grading system must be to test its reliability and reproducibility. Throughout this research, the SureView Wallace catheter has been used which consists of a soft inner lumen and a firmer co-axial component and based on this a structured grading system was designed, as has already been described in chapter 3.2 . There are different catheters on the market of a similar design which may also benefit from this stepwise approach to grade ET difficulty. At Nurture Fertility, the clinicians are taught to perform progressive steps to manoeuvre the catheter and replace the embryo as quickly and gently as possible into the uterus causing minimal trauma to the cervix and endometrial cavity.

One of the principal aims of this research was to design a clinically relevant and reliable alternative scale to grade ET ‘difficulty’. A 10-point scale (Table 3.2.2.3-1) was used to classify ET ‘difficulty’ retrospectively from clinician documentation, and this showed a strong association with IVF outcomes (chapter 3.2). This ’10-point’ graduated ET ‘difficulty’ grading scale was developed to record all the movements used by clinicians during a routine ET procedure. It is important to test the validity and reliability of any new scale such as this. This experiment has been designed therefore to test the reliability of introducing a new ET ‘difficulty’ grading scale into practice with clinicians unfamiliar with its structure.

6.1.2 Methods

6.1.2.1 Study design

In designing this experiment, a suitable animal model was found to simulate the resistance a clinician must overcome when passing an ET catheter into the uterus. It is important to have a basic understanding of the reproductive systems of different animals when selecting the best model to simulate human ET. It is well known that the structure of the cervical canal differs significantly in the female pig, compared to the sheep and cow. The structure of the cervical canal in the sow is convoluted and the least penetrable of these three animals. The sheep and bovine cervical tracts were structurally the most alike the human and allowed me to test the new grading system without recruiting human patients and obtain ethics approval. The anatomy of the cervix in the sheep is not dissimilar in structure to the human though the uterus is bicornuate. The sheep's cervical canal is like the bovine cervix only smaller in diameter and both are longer overall than the human. Whilst the bovine model offered a larger scale tract the sheep reproductive tracts were more easily attainable.

The reproductive cervical tracts of 25 sheep were obtained from the abattoir at the time of routine slaughter for meat production. Each tract contained the complete tissues of the vagina, cervix and uterus intact. Each cervical tract was dissected from the surrounding tissues and fixed to a polystyrene surface to allow minimal

movement of the reproductive tract during the simulated ET procedure and to ensure the level of difficulty remained uniform for each student. (Figure 6.1.2-1)



Figure 6.1.2-1 An example of a sheep cervical tract freshly dissected and fixed for simulated embryo transfer experiment.

Six embryology students from the University of Nottingham MSc Assisted Reproduction Techniques (ART) course and two experienced clinicians from Nurture Fertility IVF unit were invited to take part in this study. The MSc students were invited as they have learnt a degree of manual dexterity and are familiar with ART but remain inexperienced. None had prior experience of performing ET procedures.

The clinicians each had >2 year's experience of performing embryo transfers and recent clinical experience of the 10-point ET catheter grading system under trial at Nurture Fertility. The inexperienced students were given a short 30-minute tutorial

on the specific material and product characteristics of the 18-centimetre soft embryo replacement catheter (Sure View Wallace Embryo Replacement Catheter CE118; Smiths Medical) and firmer catheter (Malleable Stylet 1816ST; Smiths Medical).

During the short teaching session, all six students were shown how to use a stepwise approach to pass the ET catheter through the cervical tract. Initially they would pass the soft catheter and if resistance was too high they would next attempt to advance the co-axial outer sheath before eventually resorting to trying to overcome resistance with a malleable stylet if still unsuccessful. The students were also shown how to record their ET attempt on the ET 'difficulty' grading scale. They were given a clear explanation of how the grading system had been purposely designed in a stepwise fashion to capture the subtler movements made with the catheter when attempting to pass it though the cervix and into the uterus.

Each student was allowed 10-minutes to familiarise themselves with the catheters and scale before the experiment began for real. Each student was asked to attempt a simulated ET procedure by passing the SureView Wallace ET catheter through the cervix of each sheep model as gently as possible whilst causing minimal trauma to the cervical and uterine tissue (Figure 6.1.2-2). The student had available to them the soft SureView Wallace catheter and the firmer malleable stylet. An ET procedure was judged to be complete once it became clear from external examination of the tract that the tip of the catheter was in the mid-cavity of one of the uterine horns, or the student was unable to pass the catheter (SC6).

6.1.2.2 Condensing the 10-point ET 'difficulty' scale to a 6-point scale

A '10-point' difficulty grading scale was suggested in chapter 3.2 to record the stepwise manoeuvres undertaken by clinician to transfer an embryo (Table 6.1.2.2-1). This scale was based on the stepwise approach with a soft SureView Wallace catheter. Three progressive steps were identified to bypass resistance: (A) advance soft inner, (B) if resistance remains advance co-axial sheath past inner and (C) resort to a firm malleable stylet. The final step D involved being unable to access the uterus.

The systematic review in 'chapter 2' revealed, that deciding whether a procedure is 'easy' or 'non-easy' (difficult) is simpler than asking clinicians whether a movement has been; 'easy', involved 'some difficulty' or been 'difficult'. It therefore made sense in this experiment to minimise the potential for clinician interpretation bias by condensing the 10-point scale into a 6-point scale based on the same levels (A-D) described in 6.1.2.2

The 6-point ET 'difficulty' grading scale (SC1-6) shown in Table 6.1.2.2-2 is based on the same three principal manoeuvres performed in a stepwise fashion by clinicians, but has fewer levels to minimise subjectivity. This 6-point scale combines S1-2, S4-5 and S7-8 into single levels minimising the risk of clinician interpretation bias. This experiment set out to test the reliability of this stepwise graduated 6-point scale and potentially test the 10-point scale later if considered necessary.

Table 6.1.2.2-1 The 10-point ET 'difficulty' grading scale based on a 'soft' approach with a SureView Wallace ET catheter catheter, with manoeuvre levels (A-D)

Standard Approach (S) – 'soft catheter'		
A	**S1**	Easy / no resistance / no bend in catheter
	S2	Easy / some resistance / bend in catheter
B	**S3**	Resistance / outer advanced / easy
	S4	Resistance / outer advanced / some difficulty
	S5	Resistance / outer advanced / difficult
C	**S6**	Resistance / resort to malleable / easy
	S7	Resistance / resort to malleable / some difficulty
	S8	Resistance / resort to malleable / difficult
	S9	Resistance / resort to malleable / extra manoeuvres
D	**S10**	Unable to gain access

*Table 6.1.2.2-2 The 6-point ET 'difficulty' grading scale (SC1-6) for a 'soft' approach with a SureView Wallace ET catheter, with equivalent 10-point scale grades (S1-10) and levels (A-C). *S9 not included due to risk of tissue destruction with repeated tenaculum use.*

6-point		Soft catheter approach	10-point
A	**SC1**	Easy / minimal resistance	S1-2
B	**SC2**	Outer advanced due to resistance / easy	S3
	SC3	Outer advanced due to resistance / some difficulties	S4-5
C	**SC4**	Resistance not overcome / resort to malleable / easy	S6
	SC5	Resistance not overcome / resort to malleable / some difficulties	S7-8*
D	**SC6**	Resistance not overcome / unable to access	S10

6.1.2.3 Determinability of inter-observer variability

Six students were asked to attempt a simulated ET procedure on 25 sheep cervix models of varying difficulty. After each procedure, each student rated the movements (SC1 – 6) that they had used to pass the ET catheter on a six-point ET 'difficulty' scale (Table 6.1.2.2-2). In real time two ET clinicians familiar with this grading scale observed the student's performance and rated the difficulty of the ET procedure based on the movements that they observed the student make with the catheter. The students participating in this study were asked to perform the 25 ET procedures, divided into 5 separate blocks of 5 attempts to avoid attempting all procedures consecutively leading to fatigue or perhaps gaining from a 'learning' effect.

6.1.2.4 Statistical analysis

Statistical analysis was carried out using Stata 14 to determine the inter-observer variability between the ratings given by the subjects and clinicians performing and observing simulated ET respectively in the sheep tract. Fleiss-kappa statistic was generated to evaluate and describe the level of agreement between the clinicians and students who had assigned difficulty ratings to the ET procedure. This measure is ordinarily used to assess the level of agreement between raters when evaluating data and assigning categorical ratings to several items.



Figure 6.1.2-2 Study participant attempting to pass the soft SureView Wallace ET catheter through one of the sheep cervix models

Table 6.1.2.4-1 Suggested descriptions of Kappa values – levels of agreement (Landis and Koch, 1977)

Kappa value	Level of agreement
0.10-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1.00	Almost perfect

6.1.3 Results

All six students completed a simulated mock-ET procedure on each of the 25 cervical tracts in five separate blocks and rated their interpretation of the level of difficulty (SC1-6). Both clinicians also independently rated the difficulty of the observed procedure for each candidate performance.

6.1.3.1 Inter-observer reliability

Overall there was good agreement between the six student raters and the clinician raters' A and B. Kappa scores calculated showed 'substantial' or 'almost perfect' agreement across the 25 ET procedures rated by the students (Table 6.1.3.1-1). When the kappa value was calculated for student 3 it only showed 'moderate' agreement with the clinicians, and when you examine the raw tables of data (Table 0-1 & Table 0-2 in appendix) this student on 7 occasions rated an SC1 transfer procedure as SC2.

Table 6.1.3.1-1 Kappa values and 95% CI reflecting levels of agreement between six students and clinician's A & B – Inter-observer consistency of rating ET 'difficulty'

Student	**Clinician A**	**Kappa** *(95% CI)*	**Clinician B**	**Kappa** *(95% CI)*
1	substantial	0.79 (0.86 - 0.95)	Almost perfect	0.95 *(0.94 - 0.96)*
2	Almost perfect	0.94 *(0.85 - 0.97)*	Almost perfect	0.94 *(0.91 - 1.00)*
3	Moderate	0.56 *(0.33 - 0.62)*	Moderate	0.56 *(0.43 - 0.61)*
4	substantial	0.78 *(0.68 - 0.93)*	substantial	0.78 *(0.67 - 0.83)*
5	Almost perfect	0.98 *(0.94 - 1.00)*	Almost perfect	0.98 *(0.92 - 1.00)*
6	Almost perfect	0.90 *(0.85 - 0.96)*	Almost perfect	0.96 *(0.93 - 0.98)*

6.1.3.2 Intra-observer agreement

The levels of agreement between the six student raters who assigned a difficulty grade to each simulated model was analysed using Fleiss-kappa statistic (Table 0-3). The six student raters were all inexperienced and received the same training and there was a marked difference between the student's rating of 'difficulty'. Overall the students showed 'fair' levels of agreement with kappa 0.290 ($p < 0.0001$) suggesting poor intra-observer reliability. In general, when an ET was 'easy' all the students found this to be the case and graded the ET procedure accordingly. The kappa value was 0.647 for 'easy' procedures that had been graded at the SC1 level of difficulty, suggesting 'substantial 'agreement between the six students. However, when the students found the procedure was 'non-easy' there was marked discordance between their abilities to pass the catheter and variable levels of agreement about the difficulty of a procedure. Even though the students were

grading their movements accurately, as was evidenced by the good inter-observer agreement, they all used different techniques.

6.1.4 Discussion

The 6-point ET 'difficulty' scale has been found here to be both reliable and quick to adopt in a group of inexperienced novices. This scale does not include the 'dual loading' technique (Malleable approach) as is outlined in the 'Standard (S1-10) or Malleable (M1-5)' approach in Table 3.2.2.3-1. This 'dual-loading' approach and grading system would not have been possible to test in this sheep model as the novices had no way of predicting difficulty. A separate experiment with students using just the 'dual loading' technique in all cases and 'malleable' grading scale is needed to test this correctly. This was not deemed necessary at this stage.

It is of interest to note the wide range of grades allocated by candidates when ET procedures were 'non-easy'. This might be explained by the fact that the candidates were not experienced clinicians and so their technique to overcome resistance was not refined. Certainly, it was noted that may of the novices had significantly different approaches to overcoming difficulty and resistance. Observing the students as one of the experienced clinicians, it was clear that there were two types of reaction by the students to the procedure being 'non-easy'. The first was to react by simply pushing harder with the catheter and the second approach was to gently manipulate the catheter by changing angulation and direction of pressure. The results were clear to me that simply increasing forwards pressure rarely helped and often let to more

difficult procedures. The soft gentle manipulation approach tended to be less difficult. This is an important point as it highlights the potential challenges one faces when training new clinicians to perform ET, and the risks this presents to the embryo. To understand if this result was entirely due to the inexperience of the students will require this experiment to be repeated with 6 clinicians to see if the same effect occurs.

It seems logical though that in the hands of an experienced clinicians this simple scale (SC1-6) should be easy to introduce and be equally reliable when tested in the clinic. The next step should be to test a scale like this in the human and assess the reliability of either this condensed '6-point' scale or the earlier described '10-point' scale. This could be achieved easily by asking two experienced clinicians to observe all ET procedures taking place in theatre and grade the clinician's movements with the catheter in a similar way as has been tested here in the sheep model, and calculate the inter-observer reliability.

6.1.5 Conclusions

The '10-point' ET difficulty grading scale has been in use at Nurture Fertility since October 2015 and forms part of a 12-month period of data collection, the results of which are drawn together with ETT in the final chapter of this thesis. The reliability of a stratified scale has been tested here and even in the most inexperienced hands is can be quick to adopt and use reliably. The last and final step of this research is to

prospectively test whether combining a stratified grading scale with ETT is a more accurate alternative way to grade the ET procedure and predict IVF outcomes.

6.2 EXPT 2: IMPACT OF A NEW GRADING SYSTEM ON IVF OUTCOMES

6.2.1 Introduction

In this thesis, the high levels of heterogeneity in defining and grading ET 'difficulty' have been highlighted. One of the aims of this work was to find a new and improved grading scale suitable for clinical use. As shown in Chapter 3 a stepwise '10-point' grading system (Table 3.2.2.3-1) is a systematic way to record all extremes of ET 'difficulty' in detail and how this affects pregnancy and clinical pregnancy rates when grouped as described in 6.1.2.2 . A shortened '6-point' version of this scale was shown in chapter 6.1 to be highly reliable and easily interpreted by even the most inexperienced clinicians.

This research has also demonstrated that the ETT is an important factor during ET that directly affects IVF outcomes independent of embryo quality. It has been suggested in Chapter 5 that the cooling of culture media during ET may be affecting the embryo's potential to hatch effectively and implant. Although the time taken to transfer an embryo depends on the clinician's skill and speed, ETT is a simple scale that can be applied in any clinic. Increased time with prolonged ET procedures may be a more sensitive way to determine the outcome of ET procedures than using a stratified '10-point' grading scale.

This final experiment aims to consider both the '10-point' grading scale and the ETT together in a combined model when looking at IVF outcomes. 'Difficult' ET and ETT

are undoubtedly linked, but the question remains as to which of these is ultimately the most important factor affecting the embryo. The ’10-point’ grading system (S1-10) described in Table 3.2.2.3-1 includes a further ‘6-point’ system (M1-6) used by clinicians attempting the dual-loading technique. Since clinicians use different techniques to replace embryos it is important to consider both the ‘standard’ and ‘malleable’ techniques here in the context of ETT when studying IVF outcomes.

6.2.2 Methods

6.2.2.1 Study design

All fresh single blastocyst transfers undertaken at Nurture fertility were studied in 2016. Included in the study group was any patient that had a single fresh grade A or B blastocyst ET following IVF or ICSI treatment. Ovarian stimulation was performed with gonadotrophins (Gonal F or Menopur) and pituitary supression with GnRH analogues (Buserilin or Cetrotide). In all cases, the hCG trigger used was intramuscular pregnyl 10,000iu or Ovitrelle 250mcg subcutaneously and luteal support was with vaginal cyclogest pessaries. Patients who had treatment with donor eggs were excluded from the analysis.

Six-clinician’s working at Nurture during the study period were asked to record the level of ET difficulty for every single fresh blastocyst transfer based on the catheter approach and manoeuvres undertaken to pass the ET catheter (Table 3.2.2.3-1). Each clinician’s key performance indicators (KPI) for pregnancy and clinical

pregnancy rates were recorded to ensure they were meeting the clinic's minimum standard. All ET procedures were completed with trans-abdominal ultrasound guidance in the ET theatre adjacent to the main laboratory. All clinicians performed the ET procedure with the patient in lithotomy position after visualising the cervix with a cusco speculum and removing cervical mucus. All ET procedures were undertaken using the 18-cm SureView Wallace catheter but clinicians could also use the malleable stylet (Smiths Medical) if required.

Three months prior to starting data collection in January 2016 the '10-point' standard (S1-10) and '6-point' malleable (M1-6) embryo grading scale was introduced into routine practice, and all clinicians were familiar with its structure and used it routinely. Four groups of 'difficulty' were established based on the ET catheter manoeuvres described on these two grading scales: (A) 'Easy' = S1-2; (B) 'Medium' = S3-5; (C) 'Difficult' = S6-9 and a further grade (M) 'Malleable' = the stylet 'dual–loading' approach (Table 6.2.2.1-1).

Patient characteristics (age, smoking, and cycle number, peak estradiol level, the number of stimulation days before egg collection) were monitored. Embryo quality was determined using the simplified blastocyst grading system (Table 3.1.2.2-2), which has been shown to be a reliable and reproducible alternative to the Gardner system for grading embryos and assessing their prognostic potential (Richardson et al., 2015). The ETT as defined earlier in 4.1.2.2 was recorded by the embryologist. Clinicians recorded ET 'difficulty' using the scale described but were also asked to measure the utero-cervical angle, the endometrial thickness, if there was blood on

the catheter and if the embryos were unloaded due to a difficult transfer. Primary outcomes were pregnancy and clinical pregnancy rates. Pregnancy rate was defined as a positive urinary pregnancy test on day 13 following blastocyst transfer. Clinical pregnancy was defined as a fetal heartbeat seen on ultrasound at six weeks' gestation and live birth as the birth of a live baby after 24 weeks' gestation.

Table 6.2.2.1-1 Groupings (A-C and M) based on the '10-point' (S1-10) and '6-point' (M1-6) grading scales used to systematically categorise ET procedure 'difficulty'

	Standard Approach (S) – 'soft catheter'	
A	**S1**	Easy / no resistance / no bend in catheter
	S2	Easy / some resistance / bend in catheter
B	**S3**	Resistance / outer advanced / easy
	S4	Resistance / outer advanced / some difficulty
	S5	Resistance / outer advanced / difficult
C	**S6**	Resistance / resort to malleable / easy
	S7	Resistance / resort to malleable / some difficulty
	S8	Resistance / resort to malleable / difficult
	S9	Resistance / resort to malleable / extra manoeuvres
	S10	Unable to gain access
	Elective Malleable (M) – 'dual-loading'	
M	**M1**	Malleable / easy / no resistance
	M2	Malleable / minimal resistance
	M3	Malleable / some difficulty
	M4	Malleable / difficult
	M5	Malleable / difficult / extra manoeuvres
	M6	Unable to gain access

6.2.2.2 Statistics

Statistical analysis was carried out with SPSS 23 (Statistical Package for Social Sciences; IBM, Chicago, IL, USA). Continuous data distribution was checked by visual inspection of histograms and a student t-test or Mann-Whitney u-test used for further analysis. A one-way analysis of variance (ANOVA) was performed to check for significance of cycle characteristics across the groups and post hoc analysis where required. All primary outcome data was categorical and assessed using crosstabs analysis and chi-squared test of significance. A difference was statistically significant when $P<0.05$. A multivariate analysis was undertaken using binary logistic regression calculating odds ratios and confidence intervals. Hosmer and lemeshow values were calculated to assess the goodness of fit of the model to the data.

6.2.3 Results

218 fresh single ET procedures were undertaken during the 12-month study period and formed the study group. Clinicians used the 'soft catheter' approach in 68% of cases and the 'dual loading' technique in 32% of cases after electively passing a malleable stylet to overcome resistance. All included ET procedures were separated into the four groupings for analysis: (A) 'Easy' - 55/218 (25.2%), (B) 'Medium' - 63/218 (28.9 %), (C) 'Difficult' - 31/218 (14.2%) and (M) 'Malleable' - 69/218 (31.7%).

There was no significant difference across the groupings for most patient and cycle characteristics (smoking status, mean cycle number, peak E2, number of days to egg collection, endometrial thickness and utero-cervical angle). However, age was significantly higher across the groups ($p = 0.016$) and a Bonferroni post hoc analysis of this difference showed that the mean age in the 'malleable' group was significantly higher than in the 'easy' group. The ETT was prolonged for more difficult ET procedures and found to be significantly different across the groups ($p < 0.001$). Significant differences across the groups was noted for the number of embryos unloaded and it was found to be highest in the 'difficult' group ($p < 0.001$) and the presence of blood on the catheter was highest in the 'malleable' group ($p = 0.036$) (Table 6.2.2.2-1).

Pregnancy and clinical pregnancy rates fell as difficulty increased (A-C) with a small rise in clinical pregnancy rates in Group M, suggesting a slightly higher pre-clinical miscarriage rate in Group C (Figure 6.2.3-1). Group M represents the 'dual-loading' technique of transferring embryos, and has been included in this analysis for the purposes of examining IVF outcomes for all ET techniques and to test the actual validity of using this scale. In Group M, the different levels of difficulty (M1-6) are based on how long it takes to correctly site the malleable stylet, and although the 'dual-loading' technique had been chosen by the clinician for suspected 'difficult' ET, 53.6% of procedures were graded M1 and hence were easy. Whereas 46.4% of the procedures in the group (M2-5) involved some degree of increased difficulty.

Table 6.2.2.2-1 Patient and cycle characteristics for patients in study according to condensed 'difficulty' groupings taken from '10-point (S1-10) and '6-point' (M1-6) grading scales.

ET difficulty	A 'Easy'	B 'Medium'	C 'Difficult'	M 'Malleable'	*P value*
n =	55	63	31	69	
Age (years)	32.06 ± 3.68	32.26 ± 4.19	31.93 ± 5.04	34.18 ± 4.84	0.016
Non-smokers (%)	100	100	100	100	-
Mean Cycle No.	1.16 ± 0.6	1.10 ± 0.3	1.10 ± 0.4	1.30 ± 0.7	0.194
Peak E2	6633 ± 3596	7007 ± 4110	6928 ± 4112	8096 ± 3913	0.226
Egg collection day	14.8 ± 1.6	14.5 ± 1.2	14.0 ± 1.5	14.4 ± 1.5	0.614
Endometrial thickness (mm)	10.3 ± 2.5	11.1 ± 2.0	11.3 ± 2.0	10.8 ± 2.3	0.159
UCA (degrees)	33 ± 26	31 ± 28	25 ± 30	47 ± 36	0.359
ETT (secs)	91 ± 24	114 ± 26	255 ± 116	142 ± 169	< 0.001*
Embryo quality (%) • Grade A • Grade B	 61.8 38.2	 58.7 41.3	 51.6 48.4	 58.7 41.3	0.831
Embryos unloaded (n=)	0	0	5	1	< 0.001*
Blood on catheter (n=)	0	1	2	7	0.036*
Tenaculum usage (n=)	0	0	0	0	-

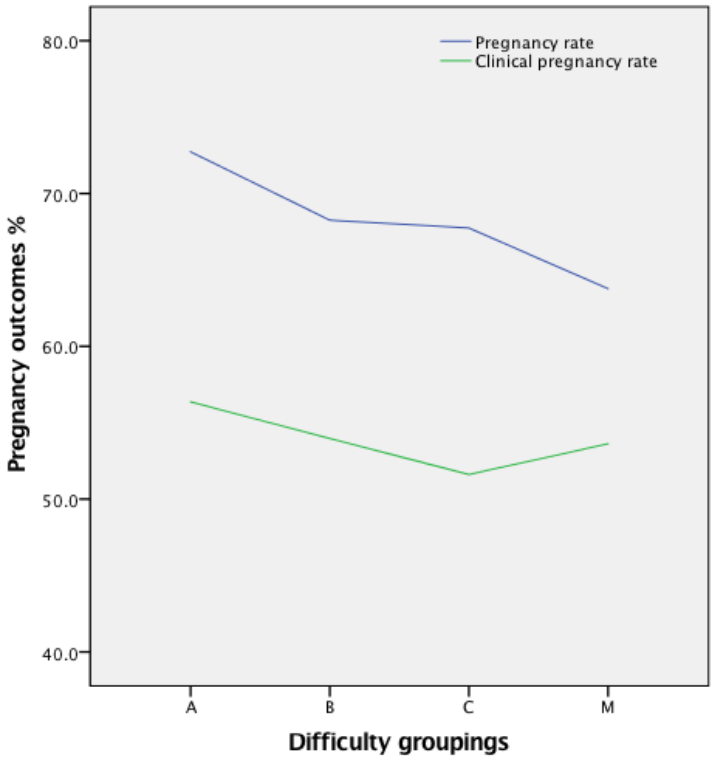


Figure 6.2.3-1 Pregnancy and clinical pregnancy rates according to changes in ET 'difficulty' in four groups (A - 'easy', B - 'Medium', C - 'Difficult' and M - 'Malleable')

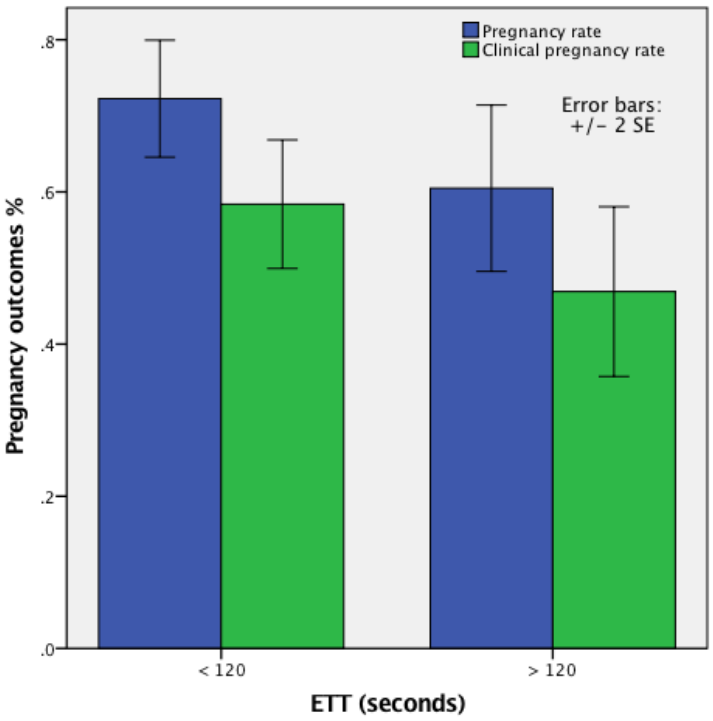


Figure 6.2.3-2 The effect of ETT on pregnancy and clinical pregnancy rates based on whether ET procedures take < 120 seconds to perform

The previous analysis in Chapter 4 showed that ETT categorised into 'two-groups' (2-ETT groups): (a) < 120 seconds and (b) > 120 seconds proved to be a highly significant model to predict pregnancy outcomes. Hence for the purposes of defining a reliable grading system that is easy to interpret this 2-ETT model was examined in this smaller prospective data set. Crosstabs analysis for this '2-ETT- group' variable showed that pregnancy rates fell from 72.3% to 60.3% (p = 0.072) and clinical pregnancy rates fell from 58.4% to 48.9% (p = 0.100). In agreement with 4.1.3.3 there was no significant interaction between embryo grade and ETT (p = 0.249)

6.2.3.1 Combining ETT and ET 'difficulty'

ET 'difficulty' and ETT in this study group were examined together in a single binary logistic regression model to assess the combined impact of these variables on pregnancy and clinical pregnancy rates, adjusting for the important confounders that may impact on implantation. The multivariate analysis did not see a significant change in pregnancy of clinical pregnancy rates when examined according to the grading scale of ET 'difficulty', however there was a significant fall in pregnancy rates when ET procedures lasted > 120 seconds (Table 6.2.3.1-1). An embryo was 56% less likely to implant and 57% less likely to reach a clinical pregnancy if the ETT was > 120 seconds. This suggests that ETT > 120 seconds is highly significant in predicting lower pregnancy rates (p = 0.044) and clinical pregnancy rates (p = 0.031).

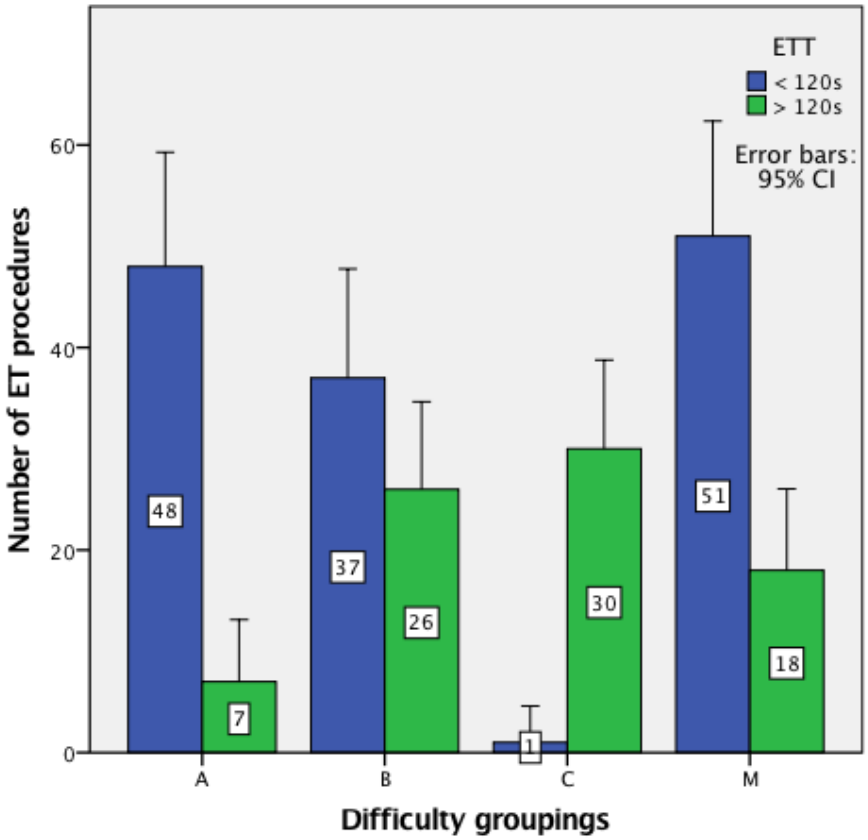


Figure 6.2.3-3 The number of ET procedure lasting > 120 seconds within different ET 'difficulty' groupngs (A – Easy, B – Medium, C – Difficult, M- Malleable)

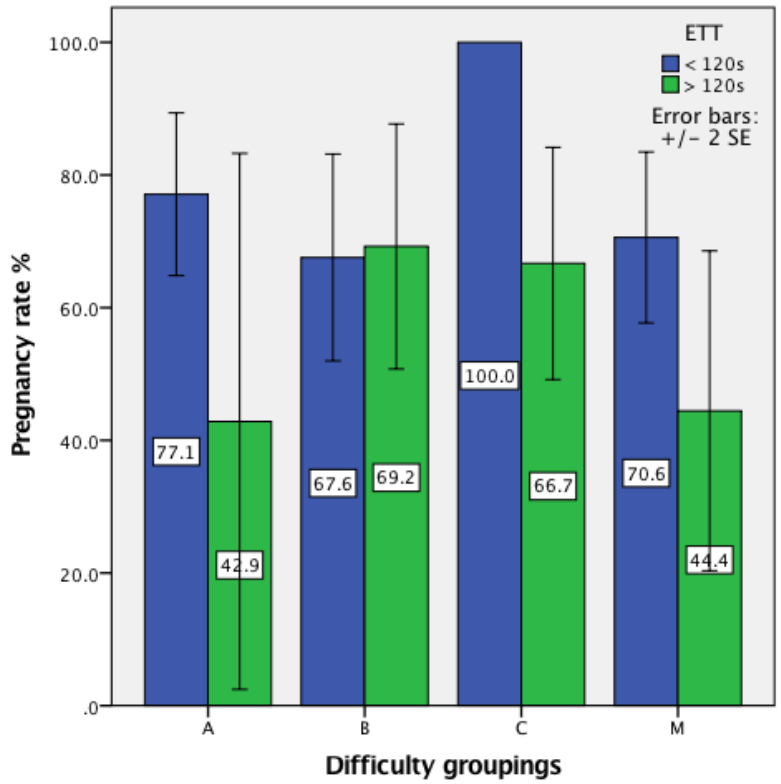


Figure 6.2.3-4 The effect of an ETT > 120 seconds on pregnancy rates within different ET 'difficulty' groupngs (A – Easy, B – Medium, C – Difficult, M- Malleable)

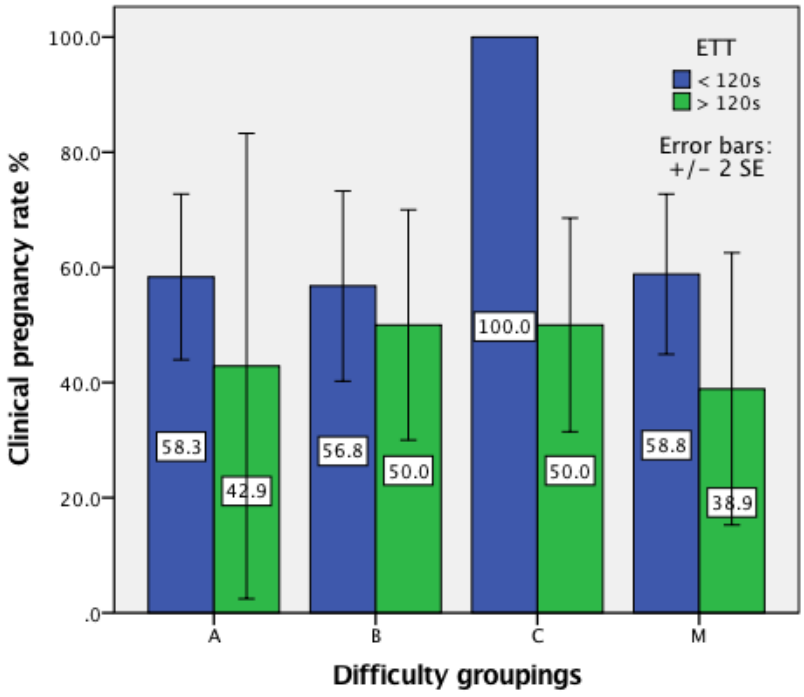


Figure 6.2.3-5 The effect of an ETT > 120 seconds on clinical pregnancy rates within different ET 'difficulty' groupngs (A – Easy, B – Medium, C – Difficult, M- Malleable)

Table 6.2.3.1-1 Multivariate analysis of ETT and ET 'difficulty' and the impact on pregnancy and clinical pregnancy rates adjusted for covariates (total cycle number, age, days to egg collection, AMH level, endometrial thickness, ET difficulty levels, blood on the catheter and embryo unloading) with odds ratios (±95% CI)

Pregnancy	**Odds ratio**	**95% C.I. for odds ratio**		**P- value**	**Odds ratio (adjusted)**	**95% C.I. for odds ratio**		**P- value**
		Lower	Upper			Lower	Upper	
Time				0.045				0.044
ETT < 120 seconds	1				1			
ETT > 120 seconds	0.50	0.25	0.99		0.44	0.20	0.98	
Difficulty				0.992				0.884
Clinical pregnancy	**Odds ratio**	**95% C.I. for odds ratio**		**P- value**	**Odds ratio (adjusted)**	**95% C.I. for odds ratio**		**P- value**
		Lower	Upper			Lower	Upper	
Time				0.087				0.031
ETT < 120 seconds	1.0				1			
ETT > 120 seconds	0.56	0.29	1.09		0.43	0.20	0.93	
Difficulty				0.930				0.993

6.2.4 Discussion

This research suggests that an ETT lasting longer than 120 seconds is a far more sensitive way to determine if the embryo will implant compared to using a clearly stratified ET difficulty grading system. It is important to note that ETT increases with increasing difficulty and a fall in pregnancy rates was seen with both variables. However, time appears to be a better delineator of pregnancy outcomes than systematically designed descriptive scale, such as the '10-point' and '6-point' scales described earlier and tested in this chapter (Table 6.2.2.1-1). There are limitations in this prospective data analysis of ETT and ET 'difficulty' though, as the numbers of procedures considered are limited across the groups. A larger study sample would likely serve to strengthen the claims made here that ETT is more sensitive at predicting IVF outcomes than a descriptive grading scale.

Whilst ETT is easy to measure and a sensitive predictor of IVF outcomes it seems sensible to consider how this could fit into a standardised definition of ET difficulty. On the strengths of the data collected in Chapter 4 and in this chapter too it is necessary to consider the '120-second' window as an important marker that changes an embryos potential to implant. Any future definition of ET 'difficulty' that is approved should consider 120 seconds as an essential factor. Chapter 2 set out to look at the prevalence of ET 'difficulty' and found the literature to contain significant heterogeneity and no consensus or a clear definition for clinical use. This work has now reached a point where an improved definition of ET difficulty can be suggested and a scale that is helpful to clinicians can be proposed.

6.2.4.1 Defining ET 'difficulty'

As ET 'difficulty' increases the ETT is more likely to be prolonged > 120 seconds (Figure 6.2.3-3). The 'dual-loading' technique tends to result in shorter ET procedures that last less than 120 seconds. In general, there appeared to be a fall in pregnancy and clinical pregnancy rates at each level of difficulty based on whether the ET procedure lasted > 120 seconds (Figure 6.2.3-4 and Figure 6.2.3-5). The total numbers of ET procedures performed in each group were small but suggest that combining a descriptive 'difficulty' grading scale with ETT may strengthen the ability to predict the chances of pregnancy and clinical pregnancy. It is suggested that future investigators consider a new definition of ET difficulty based on these results. A new clinically helpful definition might be:

"A difficult embryo transfer is any procedure where the ET catheter meets resistance and the time taken to transfer that embryo takes longer than 120 seconds as additional manoeuvres are performed to overcome that resistance."

6.2.4.2 Grading ET 'difficulty'

These results would indicate that the design of any future ET 'difficulty' grading system must be modelled on both ETT and a stratified ET difficulty grading scale. It is important first though to reconsider the purposes of a grading scale for ET procedures. In the clinical environment, the importance of having a reliable user friendly scale is to accurately capture as much information as possible about the

procedure and record systematically a very subjective measurement consistently in the clinical notes. It is also an important goal of the scale to provide as much useful information to future clinicians performing the same procedure and hopefully avoid difficulty. However, the scale should be fit for consistent use between different clinics using different catheters. The two main techniques that are used to transfer embryos are the standard 'soft catheter' approach and the 'dual-loading' firmer catheter method.

In the research environment, it is important to be able to compare studies between different clinics, using different catheters but with a universally applicable scale. It appears from this research that ETT is probably the most sensitive way to report ET 'difficulty' and so this should be a key feature in developing this improved scale. Several different 'difficulty' grading scales have been outlined and tested in this research project so far but now it is possible to combine a stratified grading scale with ETT. The next step will be to develop a clinically 'user-friendly' scale for clinical use allowing for different techniques and different catheters.

6.2.4.3 A new ET 'difficulty' grading scale

The soft catheter approach (S1-10) and 'malleable' or 'dual loading' approach (M1-6) have many levels which are useful for clinicians aiming to accurately report difficulty, but for research purposes combining these levels and considering ETT at the same time is far more critical for clinicians evaluating IVF outcomes. It is suggested that the system outlined in Table 6.2.4.3-1 be introduced into clinical practice and the

outcomes be studied prospectively. It is important that the difficulties encountered during the dual-loading technique are acknowledged on this scale which is why the M 'grade has been split into 'MA' and 'MB'. The information identified in this study in Chapter 6.2 suggests that the likelihood of surrogate markers of difficulty (such as blood on the catheter) occurring is greater with a 'difficult' malleable stylet ET.

Table 6.2.4.3-1 A new ET 'difficulty' grading system for both clinical and research purposes that combines a systematic stepwise grading scale with ETT. Clinically there are two scales on which to record procedures (S1-10 and M1-6) and which can easily be translated from 'bedside to bench' to allow more accurate comparisons.

Research		**Clinical**	
< 120 (secs)	**> 120 (secs)**	**Standard Approach (S) – 'soft catheter'**	
SA	**SA2**	**S1**	Easy / no resistance / no bend in catheter
		S2	Easy / some resistance / bend in catheter
SB	**SB2**	**S3**	Resistance / outer advanced / easy
		S4	Resistance / outer advanced / some difficulty
		S5	Resistance / outer advanced / difficult
SC	**SC2**	**S6**	Resistance / resort to malleable / easy
		S7	Resistance / resort to malleable / some difficulty
		S8	Resistance / resort to malleable / difficult
		S9	Resistance / resort to malleable / extra manoeuvres
		S10	Unable to gain access
		Elective Malleable (M) – 'dual-loading'	
MA	**MA2**	**M1**	Malleable / easy / no resistance
		M2	Malleable / minimal resistance
MB	**MB2**	**M3**	Malleable / some difficulty
		M4	Malleable / difficult
		M5	Malleable / difficult / extra manoeuvres
		M6	Unable to gain access

6.2.5 Conclusions

This final chapter has confirmed with a prospective study that ETT is a more accurate way to predict the IVF outcomes of 'difficult' ET than using a descriptive scale as has been used throughout the literature. As discussed in Chapter 2 the significant heterogeneity across the literature in defining and grading ET has made it very challenging to draw meaningful conclusions. This new scale suggested here can be used in all clinics by all clinicians replacing embryos and is highly likely to accurately predict IVF outcomes.

Chapter 7

General discussions & conclusions

7.1 SUMMARY OF RESEARCH FINDINGS

7.1.1 What exactly do we mean by 'difficult' ET and how common is it?

When I started this thesis, I identified that there were several studies examining the effects of ET on IVF outcomes. This final important step of an IVF cycle had been studied extensively and many of the factors that influence the ease of ET had been investigated over the years. However, a fundamental issue lay unanswered, because there was no consensus on how best to describe and define ET 'difficulty'. Indeed, some studies regarded 'difficult' ET to be so uncommon that only 0.4% of patients experienced it, whilst other studies claimed that 29.5% of patients experienced a difficult ET procedure (Matorras et al., 2002, Tiboni et al., 2012a). As a result, there appeared to be a lack of understanding about how to practically define 'difficult' ET. I consequently set out to estimate the prevalence of 'difficult' ET from the existing literature, with a systematic review and attempt to find an improved definition for this subjective measurement.

There is significant heterogeneity in the ET literature with the way 'difficulty' is described and defined. I could not find a universal definition in use to describe 'difficult' ET. At the very start of this thesis I could only conclude following this systematic review that a difficult ET was, *"…a procedure where the ET catheter will not pass easily requiring additional manoeuvres to replace the embryo".* This was based on numerous studies reporting 'difficulty' over multiple levels and all

associated with the ET catheter meeting ‘resistance’ and requiring manipulation or manoeuvres to pass.

At the end of thissystematic review it was established that the prevalence of ‘difficult’ ET lies somewhere between 3.7% - 16.9%, and is hugely influenced by how it is defined and graded. I therefore set out to improve the current definition of ET ‘difficulty’ and design an improved scale to grade and describe it.

During this systematic review of the literature it became evident that there was no consistency in the existing grading systems used to describe ET ‘difficulty’. I included two studies by the same author that used a slightly different ET ‘difficulty’ grading scale (Sallam et al., 2002, Sallam et al., 2003), when I would have expected some consistency. I looked at all the current systems in use and realised that a consensus was needed and that something different was needed to replace the current scales.

7.1.2 How best can we measure ‘difficult’ ET?

IVF treatment is tightly regulated yet clinical practices differs noticeably around the intricacies of protocols and procedures. The ET is one such area of treatment that has very little agreement about the best way to perform and measure it. The ‘ease’ of the ET procedure depends on many factors but crucially the clinician’s individual skill and experience remain central to this. As a result, at times the ET procedure can appear as more of a ‘dark art’ than a clinical science. Having reviewed the literature

in this area I discovered that without a clear definition of ‘difficult’ no grading system could truly be fit for purpose.

I began to search for a more universally applicable ET ‘difficulty’ grading system that was different to the current definitions and grading scales. A visual analogue scale was simple to use clinically but no more accurate or reliable at establishing the difficulty of an ET procedure than traditional clinician narrative. This was probably because the same inter-user interpretation of ‘difficulty’ that affects a clinician’s subjective description of a procedure also causes wide variation in clinician scoring when using a basic VAS tool.

Whilst many different grading scales of ‘difficulty’ exist very few had considered the lesser manoeuvres a clinician makes to gently manipulate the ET catheter and pass resistance. Most levels of difficulty were established around the occurrence of the major surrogate markers of ‘difficulty’ such as blood on the catheter, use of a tenaculum or the need to dilate the cervix. I therefore developed a systematic scale to measure ‘difficulty’ and include both ends of the spectrum from the lesser difficulties encountered to the major problems that occur. I then tested this stepwise grading scale retrospectively in a fresh single ET study and found it to be accurate at predicting pregnancy outcomes. ET ‘difficulty’ graded in this way showed good correlation with IVF outcomes, and in agreement with the existing literature the pregnancy and clinical pregnancy rates fell with increasing difficulty.

7.1.3 Why does ‘difficult’ really matter?

Throughout the literature descriptive scales of difficulty were common, yet there had been only a few attempts to evaluate why ‘difficulty’ makes a difference to outcome. Studies had suggested that blood on the catheter may be detrimental to embryos or that using a tenaculum may increase embryo expulsion by increasing myometrial contractions. However, these explanations could not explain the findings that even the lesser difficulties can reduce pregnancy and clinical pregnancy rates. One clear association noted with the ET procedure was that difficult procedures tended to take longer to complete and it was this that changed the focus on unravelling why a ‘difficult’ ET affects an embryos potential to implant. There was conflicting evidence in the literature regarding ETT and its importance to IVF outcomes. It remained unclear whether ETT affects embryos of different qualities equally or whether the effect is dependent on the quality of that embryo.

I set out to test whether ETT was an important marker of ‘difficulty’ that directly impacts on a single embryos potential to implant. In a study of ETT (Chapter 4), I established that there was a strong association with increasing ETT and falling pregnancy and clinical pregnancy rates. Importantly though this also showed that this effect was independent of the quality of the embryo being transferred and so affected all embryos equally irrespective of their quality.

7.1.4 Why is the ETT factor important?

I had established that ETT can have a significant impact on pregnancy and clinical pregnancy rates resulting from fresh single ET cycles. However, I still had no deeper understanding of why this might be the case and so set out next to try and understand the mechanism driving this. First by addressing the laboratory procedures, it became clear that the significant factor to change during prolonged ET procedures was that the culture media cooled significantly, potentially destabilising the embryo's microenvironment and affecting the embryos potential. This observation required further investigation though and so I began to look at mouse embryos in time-lapse photography and see if a simulated prolonged ETT affected morphokinetic parameters.

Morphokinetics seemed the logical place to direct my focus next and to study the developing mouse embryo after stress in time lapse photography. Whether any significant morphokinetic changes occurred in the mouse embryo that may be reflective of embryo cooling during a prolonged ET was of interest. This time-lapse study provided some interesting pilot data for consideration but was limited by the fact that experiments were undertaken in a mouse model and the embryo numbers were low. It did reveal a possible issue with embryo hatching that requires further work to elucidate whether a prolonged ETT affects an embryo's hatching potential.

This work has demonstrated how the ETT is a clinically important factor. It has been hypothesised that if the ETT is less than 120 seconds this may improve the chances

of successful implantation occuring. The ‘standard’ ET approach may result in a difficult transfer and prolong the length of time the embryos are in the catheter and exposed to cooling and potential damage. However, the ‘dual-loading’ ET technique may well offer an advantage to the clinician as this approach tends to result in shorter ET procedures overall, hence minimising the legth of time that the embryos are exposed to sub-optimal conditions. The reason being that the ‘dual loading’ technique introduces the outer catheter into the cervix initially and the difficulties are overcome before the embryos are loaded into the soft inner catheter. Using this ‘dual-loading’ technique embryos are loaded from the incubator, into the catheter and straight into the uterus, overall resulting in a short ETT.

7.1.5 A novel approach to defining and grading ET ‘difficulty’

The universal grading scale I have developed during this research adopts a stepwise approach and considers the many problems encountered with ‘difficult’ ET, and has been shown to be strongly linked to pregnancy outcomes. I have shown that introducing a new scale like this into clinical practice can be reliable and quick to establish even in the most inexperienced hands. The final step of this work was to draw together all the evidence in this thesis and develop a novel grading system for future use to describe and accurately record ET ‘difficulty’.

This thesis suggests the first universal ET ‘difficulty’ grading system that considers the full spectrum of catheter manoeuvres with the ETT as a key factor. It creates a

clear reporting system that potentially could be adopted into any clinic performing embryo transfer. This novel scale still requires further evaluation in a single IVF centre initially to ensure its integrity and accuracy at predicting IVF outcomes, before finally testing it in a much larger study of multiple IVF centres using different ET catheters and techniques.

7.2 FUTURE DIRECTIONS

7.2.1 Testing the 'New' ET difficulty grading system

I would like to build on the work of this thesis by establishing if the proposed novel ET 'difficulty' grading system (Table 6.2.4.3-1) does accurately predict IVF outcomes. It is the first widely applicable grading system that has been proposed that combines ETT with conventional descriptive narrative. I would like to test this grading system in a single centre initially and ultimately in a multicentre study where different catheters and different techniques are used to replace embryos.

7.2.2 Predicting ET 'difficulty'

The challenge remains that even if a consensus is agreed on how to 'define and grade' ET difficulty, we are still unable to accurately predict which patients will have a 'difficult' ET. Whilst there are strong markers that many clinicians associate with a difficult ET procedure there is no accurate model available yet to predict a 'difficult' ET without performing a mock-ET. This has not been shown to be an accurate predictor of 'difficulty' unless performed at the time of ET and this has disadvantages in terms of endometrial damage.

The reduction in pregnancy and clinical pregnancy rates that occurs as ET difficulty and ETT increases is clear to see. Therefore, it is important that a clinician can

predict a difficult ET and do everything possible to avoid it. The use of a 'malleable' stylet has been criticised in past studies (Tiboni et al., 2012b), but results from this thesis suggest it may benefit some patients. Further work is needed here, once we can accurately predict difficulty, to decide in a non-inferiority randomised controlled trial whether an elective malleable technique ('dual loading') produces higher pregnancy rates compared to the standard 'soft catheter' approach.

A study looking at the causes of difficulty is long overdue. Recent work from Larue et al has finally begun to focus on the mechanisms preventing a smooth passage of the ET catheter. In this study, the authors focus on the anatomical features of the cervix that appear to be present at difficult procedures(Larue et al., 2017). I would like to try and develop a clinically useful model that can accurately predict whether a patient will have a difficult ET. The theory is based on Larue's work and the use of ultrasound of the cervix in high definition and it is an area of work I would like to pursue further.

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Appendices

Appendix 1. Thawing Mouse Embryos

The following instructions for one-cell mouse embryo thawing is suggested by Embryotech. First, remove a single straw from storage tank and expose it to room temperature air for 2-3 minutes. Next, place the straw in a 37°C water bath for 1 minute. Remove the straw from water bath and wipe it dry. Furthermore, remove handle and by using a pair of scissors cut the straw between the lower heat seal and the column of media. Then, make a second cut to bisect the cotton plug at the polyvinyl acetate section.

In a petri-dish using the provided stylet, push down on the remaining cotton plug, expelling the contents of the straw into a drop of Hepes buffered medium. Immediately rinse the embryos into 2 additional drops of the Hepes buffered media. Allow embryos to rehydrate for 10 minutes in the final rinse droplet.

Appendix 2. The pooled prevalence of ET 'difficulty' for studies included in systematic review – all separate sub-group analyses

Study	Effect	[95% Conf.	Interval]	% Weight
Leeton et al. 1982	**76.470**	**62.918**	**90.022**	**4.38**
Wisanto et al 1989	**86.250**	**70.129**	**102.371**	**3.31**
Mansour et al. 1990	**85.070**	**69.455**	**100.685**	**3.49**
Awonuga et al 1998	**74.600**	**58.205**	**90.995**	**3.22**
Tur-kaspa et al. 199	**85.940**	**68.709**	**103.171**	**2.96**
Kan et al. 1999	**75.400**	**62.252**	**88.548**	**4.58**
Noyes et al. 1999	**75.820**	**59.343**	**92.297**	**3.19**
Garcia-velasco et al	**85.560**	**69.639**	**101.481**	**3.38**
Mattoras et al. 2002	**88.730**	**71.933**	**105.527**	**3.09**
Sallam et al. 2002	**82.340**	**65.738**	**98.942**	**3.15**
Tomas et al. 2002	**58.680**	**43.758**	**73.602**	**3.76**
Mirkin et al. 2003	**90.490**	**72.181**	**108.799**	**2.67**
Sallam et al. 2003	**80.990**	**64.287**	**97.693**	**3.12**
Spandorfer et al. 20	**95.310**	**76.582**	**114.038**	**2.56**
Mcllveen et al 2005	**84.000**	**72.084**	**95.916**	**5.31**
Flisser et al. 2006	**89.150**	**74.322**	**103.978**	**3.79**
Ciray et al. 2007	**86.000**	**70.648**	**101.352**	**3.59**
Eskander et al. 2007	**93.360**	**77.817**	**108.903**	**3.51**
Allahbadia et al. 20	**94.280**	**81.355**	**107.205**	**4.71**
Bodri et al. 2011	**94.850**	**78.736**	**110.964**	**3.31**
Singh et al. 2012	**79.570**	**64.736**	**94.404**	**3.79**
Spitzer et al. 2012	**74.970**	**58.613**	**91.327**	**3.23**
Tiboni et al. 2012	**70.340**	**54.983**	**85.697**	**3.58**
Ghaffari et al. 201	**81.440**	**64.804**	**98.076**	**3.14**
Listijono et al. 201	**92.160**	**73.478**	**110.842**	**2.58**
Yilmaz et al. 2013	**74.120**	**59.379**	**88.861**	**3.83**
Ghannem et al. 2015	**72.980**	**57.078**	**88.882**	**3.38**
Kava-Braverman et al	**91.570**	**72.926**	**110.214**	**2.58**
Plowden et al. 2017	**90.880**	**73.140**	**108.620**	**2.82**
Overall effect (dl)	**82.704**	**79.487**	**85.921**	**100.00**

Figure 7.2.2-1 Weighted prevalence and 95% confidence intervals of 'easy' embryo transfer for all included studies

Study	Effect	[95% Conf.	Interval]	% Weight
Leeton et al. 1982	5.880	1.196	10.564	3.67
Wisanto et al 1989	11.500	4.950	18.050	3.17
Mansour et al. 1990	14.930	7.527	22.333	2.94
Awonuga et al 1998	25.400	15.627	35.173	2.36
Tur-kaspa et al. 199	8.430	2.767	14.093	3.41
Kan et al. 1999	13.900	6.869	20.931	3.04
Noyes et al. 1999	17.540	9.396	25.684	2.75
Meriano et al. 2000	9.090	3.603	14.577	3.46
Garcia-velasco et al	12.030	5.342	18.718	3.13
Mattoras et al. 2002	3.689	-0.062	7.440	3.91
Sallam et al. 2002	17.660	9.538	25.782	2.76
Tomas et al. 2002	7.114	1.890	12.338	3.53
Mirkin et al. 2003	4.457	0.323	8.591	3.81
Sallam et al. 2003	19.005	10.565	27.445	2.68
Spandorfer et al. 20	4.680	0.444	8.916	3.79
Mcllveen et al 2005	4.000	0.133	7.867	3.88
Flisser et al. 2006	0.401	-0.839	1.641	4.34
Ciray et al. 2007	14.000	6.840	21.160	3.01
Eskander et al. 2007	6.643	1.650	11.636	3.59
Allahbadia et al. 20	1.714	-0.839	4.267	4.16
Bodri et al. 2011	5.150	0.737	9.563	3.74
Singh et al. 2012	19.014	10.758	27.270	2.72
Spitzer et al. 2012	3.800	-0.014	7.614	3.89
Tiboni et al. 2012	29.656	19.273	40.039	2.23
Ghaffari et al. 201	18.555	10.224	26.886	2.70
Listijono et al. 201	2.066	-0.751	4.883	4.11
Yilmaz et al. 2013	11.501	4.977	18.025	3.18
Ghannem et al. 2015	27.010	17.010	37.010	2.31
Kava-Braverman et al	7.090	1.873	12.307	3.53
Plowden et al. 2017	1.627	-0.871	4.124	4.17
Overall effect (dl)	9.561	7.356	11.766	100.00

Figure 7.2.2-2 Weighted prevalence and 95% confidence intervals of 'difficult' embryo transfer in all included studies

Study	Effect	[95% Conf.	Interval]	% Weight
Wisanto et al 1989	**11.500**	**4.950**	**18.050**	**5.55**
Awonuga et al 1998	**25.400**	**15.627**	**35.173**	**4.14**
Kan et al. 1999	**13.900**	**6.869**	**20.931**	**5.32**
Meriano et al. 2000	**9.090**	**3.603**	**14.577**	**6.04**
Garcia-velasco et al	**2.406**	**-0.624**	**5.436**	**7.09**
Mattoras et al. 2002	**3.689**	**-0.062**	**7.440**	**6.81**
Mirkin et al. 2003	**4.457**	**0.323**	**8.591**	**6.65**
Sallam et al. 2003	**19.005**	**10.565**	**27.445**	**4.69**
Flisser et al. 2006	**0.401**	**-0.839**	**1.641**	**7.56**
Ciray et al. 2007	**14.000**	**6.840**	**21.160**	**5.26**
Allahbadia et al. 20	**1.714**	**-0.839**	**4.267**	**7.24**
Spitzer et al. 2012	**3.800**	**-0.014**	**7.614**	**6.79**
Tiboni et al. 2012	**29.656**	**19.273**	**40.039**	**3.91**
Listijono et al. 201	**2.066**	**-0.751**	**4.883**	**7.16**
Yilmaz et al. 2013	**11.501**	**4.977**	**18.025**	**5.56**
Ghannem et al. 2015	**27.010**	**17.010**	**37.010**	**4.05**
Kava-Braverman et al	**7.090**	**1.873**	**12.307**	**6.17**
Overall effect (dl)	**9.216**	**6.283**	**12.150**	**100.00**

Figure 7.2.2-3 Weighted prevalence and 95% confidence intervals for all included studies reporting 'difficult' embryo transfer 'optimally'

Study	Effect	[95% Conf.	Interval]	% Weight
Leeton et al. 1982	**5.880**	**1.196**	**10.564**	**8.54**
Mansour et al. 1990	**14.930**	**7.527**	**22.333**	**6.53**
Tur-kaspa et al. 199	**8.430**	**2.767**	**14.093**	**7.80**
Noyes et al. 1999	**17.540**	**9.396**	**25.684**	**6.04**
Sallam et al. 2002	**17.660**	**9.538**	**25.782**	**6.05**
Tomas et al. 2002	**7.114**	**1.890**	**12.338**	**8.13**
Spandorfer et al. 20	**4.680**	**0.444**	**8.916**	**8.87**
Mcllveen et al 2005	**4.000**	**0.133**	**7.867**	**9.13**
Eskander et al. 2007	**6.643**	**1.650**	**11.636**	**8.30**
Bodri et al. 2011	**5.150**	**0.737**	**9.563**	**8.74**
Singh et al. 2012	**19.014**	**10.758**	**27.270**	**5.96**
Ghaffari et al. 201	**18.555**	**10.224**	**26.886**	**5.92**
Plowden et al. 2017	**1.627**	**-0.871**	**4.124**	**10.00**
Overall effect (dl)	**9.017**	**5.991**	**12.043**	**100.00**

Figure 7.2.2-4 Weighted prevalence and 95% confidence intervals for all included studies reporting 'difficult' embryo transfer 'sub-optimally'

Study	Effect	[95% Conf.	Interval]	% Weight
Wisanto et al 1989	**11.500**	**4.950**	**18.050**	**6.99**
Mansour et al. 1990	**14.930**	**7.527**	**22.333**	**6.60**
Awonuga et al 1998	**25.400**	**15.627**	**35.173**	**5.53**
Meriano et al. 2000	**9.090**	**3.603**	**14.577**	**7.47**
Sallam et al. 2002	**17.660**	**9.538**	**25.782**	**6.27**
Sallam et al. 2003	**19.005**	**10.565**	**27.445**	**6.12**
Spandorfer et al. 20	**4.680**	**0.444**	**8.916**	**8.00**
Ciray et al. 2007	**14.000**	**6.840**	**21.160**	**6.71**
Eskander et al. 2007	**6.643**	**1.650**	**11.636**	**7.69**
Bodri et al. 2011	**5.150**	**0.737**	**9.563**	**7.93**
Singh et al. 2012	**19.014**	**10.758**	**27.270**	**6.21**
Tiboni et al. 2012	**29.656**	**19.273**	**40.039**	**5.28**
Ghaffari et al. 201	**18.555**	**10.224**	**26.886**	**6.17**
Ghannem et al. 2015	**27.010**	**17.010**	**37.010**	**5.44**
Kava-Braverman et al	**7.090**	**1.873**	**12.307**	**7.59**
Overall effect (dl)	**14.275**	**10.542**	**18.007**	**100.00**

Figure 7.2.2-5 Weighted prevalence and 95% confidence intervals for all included studies reporting two-levels of 'difficult' embryo transfer

Study	Effect	[95% Conf.	Interval]	% Weight
Wisanto et al 1989	**11.500**	**4.950**	**18.050**	**13.53**
Awonuga et al 1998	**25.400**	**15.627**	**35.173**	**11.05**
Meriano et al. 2000	**9.090**	**3.603**	**14.577**	**14.31**
Sallam et al. 2003	**19.005**	**10.565**	**27.445**	**12.07**
Ciray et al. 2007	**14.000**	**6.840**	**21.160**	**13.06**
Tiboni et al. 2012	**29.656**	**19.273**	**40.039**	**10.59**
Ghannem et al. 2015	**27.010**	**17.010**	**37.010**	**10.88**
Kava-Braverman et al	**7.090**	**1.873**	**12.307**	**14.50**
Overall effect (dl)	**16.894**	**11.267**	**22.520**	**100.00**

Figure 7.2.2-6 Weighted prevalence and 95% confidence intervals for all included studies reporting two-levels of 'difficult' embryo transfer 'optimally'

Study	Effect	[95% Conf.	Interval]	% Weight
Mansour et al. 1990	**14.930**	**7.527**	**22.333**	**13.25**
Sallam et al. 2002	**17.660**	**9.538**	**25.782**	**12.43**
Spandorfer et al. 20	**4.680**	**0.444**	**8.916**	**16.96**
Eskander et al. 2007	**6.643**	**1.650**	**11.636**	**16.11**
Bodri et al. 2011	**5.150**	**0.737**	**9.563**	**16.77**
Singh et al. 2012	**19.014**	**10.758**	**27.270**	**12.28**
Ghaffari et al. 201	**18.555**	**10.224**	**26.886**	**12.20**
Overall effect (dl)	**11.499**	**6.774**	**16.224**	**100.00**

Figure 7.2.2-7 Weighted prevalence and 95% confidence intervals for all included studies reporting two-levels of 'difficult' embryo transfer 'sub-optimally'

Study	Effect	[95% Conf.	Interval]	% Weight
Kan et al. 1999	**13.900**	**6.869**	**20.931**	**4.97**
Garcia-velasco et al	**2.406**	**-0.624**	**5.436**	**11.42**
Tomas et al. 2002	**7.114**	**1.890**	**12.338**	**7.21**
Mirkin et al. 2003	**4.457**	**0.323**	**8.591**	**9.10**
Mcllveen et al 2005	**4.000**	**0.133**	**7.867**	**9.63**
Flisser et al. 2006	**0.401**	**-0.839**	**1.641**	**15.16**
Allahbadia et al. 20	**1.714**	**-0.839**	**4.267**	**12.49**
Listijono et al. 201	**2.066**	**-0.751**	**4.883**	**11.90**
Yilmaz et al. 2013	**11.501**	**4.977**	**18.025**	**5.50**
Plowden et al. 2017	**1.627**	**-0.871**	**4.124**	**12.62**
Overall effect (dl)	**3.628**	**1.746**	**5.510**	**100.00**

Figure 7.2.2-8 Weighted prevalence and 95% confidence intervals for all included studies reporting three-levels of 'difficult' embryo transfer

Study	Effect	[95% Conf.	Interval]	% Weight
Kan et al. 1999	**13.900**	**6.869**	**20.931**	**7.77**
Garcia-velasco et al	**2.406**	**-0.624**	**5.436**	**16.12**
Mirkin et al. 2003	**4.457**	**0.323**	**8.591**	**13.31**
Flisser et al. 2006	**0.401**	**-0.839**	**1.641**	**20.25**
Allahbadia et al. 20	**1.714**	**-0.839**	**4.267**	**17.35**
Listijono et al. 201	**2.066**	**-0.751**	**4.883**	**16.67**
Yilmaz et al. 2013	**11.501**	**4.977**	**18.025**	**8.53**
Overall effect (dl)	**3.765**	**1.307**	**6.223**	**100.00**

Figure 7.2.2-9 Weighted prevalence and 95% confidence intervals for all included studies reporting three-levels of 'difficult' embryo transfer 'optimally'

Study	Effect	[95% Conf.	Interval]	% Weight
Tomas et al. 2002	**7.114**	**1.890**	**12.338**	**21.86**
Mcllveen et al 2005	**4.000**	**0.133**	**7.867**	**31.69**
Plowden et al. 2017	**1.627**	**-0.871**	**4.124**	**46.45**
Overall effect (dl)	**3.578**	**0.630**	**6.526**	**100.00**

Figure 7.2.2-10 Weighted prevalence and 95% confidence intervals for all included studies reporting three-levels of 'difficult' embryo transfer 'sub-optimally'

Study	Effect	[95% Conf.	Interval]	% Weight
Leeton et al. 1982	**5.880**	**1.196**	**10.564**	**21.17**
Tur-kaspa et al. 199	**8.430**	**2.767**	**14.093**	**18.21**
Noyes et al. 1999	**17.540**	**9.396**	**25.684**	**12.36**
Mattoras et al. 2002	**3.689**	**-0.062**	**7.440**	**24.23**
Spitzer et al. 2012	**3.800**	**-0.014**	**7.614**	**24.02**
Overall effect (dl)	**6.755**	**3.124**	**10.387**	**100.00**

Figure 7.2.2-11 Weighted prevalence and 95% confidence intervals for all included studies reporting four-levels of 'difficult' embryo transfer

Study	Effect	[95% Conf.	Interval]	% Weight
Mattoras et al. 2002	**3.689**	**-0.062**	**7.440**	**50.83**
Spitzer et al. 2012	**3.800**	**-0.014**	**7.614**	**49.17**
Overall effect (dl)	**3.744**	**1.069**	**6.418**	**100.00**

Figure 7.2.2-12 Weighted prevalence and 95% confidence intervals for all included studies reporting four-levels of 'difficult' embryo transfer 'optimally'

Study	Effect	[95% Conf.	Interval]	% Weight
Leeton et al. 1982	**5.880**	**1.196**	**10.564**	**38.84**
Tur-kaspa et al. 199	**8.430**	**2.767**	**14.093**	**34.98**
Noyes et al. 1999	**17.540**	**9.396**	**25.684**	**26.18**
Overall effect (dl)	**9.825**	**3.852**	**15.798**	**100.00**

Figure 7.2.2-13 Weighted prevalence and 95% confidence intervals for all included studies reporting four-levels of 'difficult' embryo transfer 'sub-optimally'

Appendix 3. Raw tables of agreement between students and clinician raters

Table 0-1 Tables rating levels of agreement between students and clinician A

Student 1

Clin A	1	2	3	4	5	Total
1	16	0	0	0	0	16
2	0	1	0	0	0	1
3	0	1	1	1	0	3
4	0	0	0	0	1	1
5	0	0	0	0	2	2
6	0	0	0	0	0	2
Total	16	2	1	1	3	25

Student 2

Clin A	1	2	3	4	5	Total
1	17	0	0	0	0	17
2	0	1	1	0	0	2
3	0	0	1	0	0	1
4	0	0	0	2	0	2
5	0	0	0	1	1	2
6	0	0	0	0	0	1
Total	17	1	2	3	1	25

Student 3

Clin A	1	2	3	4	5	Total
1	11	7	1	0	0	19
2	0	0	0	0	1	1
3	0	0	0	2	0	2
4	0	0	0	1	0	1
5	0	0	0	1	1	2
Total	11	7	1	4	2	25

Student 4

Clin A	1	2	3	4	5	Total
1	14	0	0	1	0	15
2	0	1	0	0	0	1
3	0	2	4	0	0	6
4	0	0	0	1	1	2
5	0	0	0	1	0	1
Total	14	3	4	3	1	25

Student 5

Clin A	1	2	3	4	5	Total
1	16	0	0	0	0	16
2	0	1	0	0	0	1
3	0	0	2	0	0	2
4	0	0	0	2	1	3
5	0	0	0	0	1	1
6	0	0	0	0	0	2
Total	16	1	2	2	2	25

Student 6

Clin A	1	2	3	5	6	Total
1	13	1	0	0	0	14
2	0	2	0	0	0	2
3	0	0	2	0	0	2
4	0	0	0	4	0	4
6	0	0	0	0	3	3
Total	13	3	2	4	3	25

Table 0-2 Tables rating levels of agreement between students and clinician B

Student 1

Clin B	1	2	3	4	5	Total
1	16	0	0	0	0	16
2	0	2	1	0	0	3
3	0	0	0	1	0	1
4	0	0	0	0	0	0
5	0	0	0	0	3	3
6	0	0	0	0	0	2
Total	16	2	1	1	3	25

Student 2

Clin B	1	2	3	4	5	Total
1	17	0	0	0	0	17
2	0	1	0	0	0	1
3	0	0	2	1	0	3
4	0	0	0	2	1	3
5	0	0	0	0	0	0
6	0	0	0	0	0	1
Total	17	1	2	3	1	25

Student 3

Clin B	1	2	3	4	5	Total
1	11	7	1	0	0	19
2	0	0	0	0	1	1
3	0	0	0	2	0	2
4	0	0	0	1	0	1
5	0	0	0	1	1	2
Total	11	7	1	4	2	25

Student 4

Clin B	1	2	3	4	5	Total
1	14	0	0	1	0	15
2	0	1	0	0	0	1
3	0	2	4	0	0	6
4	0	0	0	1	1	2
5	0	0	0	1	0	1
Total	14	3	4	3	1	25

Student 5

Clin B	1	2	3	4	5	Total
1	16	0	0	0	0	16
2	0	1	0	0	0	1
3	0	0	2	0	0	2
4	0	0	0	2	1	3
5	0	0	0	0	1	1
6	0	0	0	0	0	2
Total	16	1	2	2	2	25

Student 6

Clin B	1	2	3	4	5	Total
1	13	0	0	0	0	13
2	0	3	0	0	0	3
3	0	0	2	0	0	2
4	0	0	0	0	2	2
5	0	0	0	0	2	2
6	0	0	0	0	0	3
Total	13	3	2	0	4	25

Table 0-3 The levels of agreement between students allocating difficulty grades to each sheep cervical model.

Difficulty / Sheep model	SC1	SC2	SC3	SC4	SC5	SC6	
1	0	2	1	1	1	1	Non-easy
2	6	0	0	0	0	0	Easy
3	2	2	0	1	1	0	Non-easy
4	6	0	0	0	0	0	Easy
5	6	0	0	0	0	0	Easy
6	1	1	0	1	1	2	Non-easy
7	6	0	0	0	0	0	Easy
8	0	1	2	1	0	2	Non-easy
9	6	0	0	0	0	0	Easy
10	6	0	0	0	0	0	Easy
11	1	1	3	0	1	0	Non-easy
12	1	0	0	3	2	0	Non-easy
13	1	1	2	1	0	1	Non-easy
14	5	1	0	0	0	0	Easy
15	6	0	0	0	0	0	Easy
16	2	1	1	0	1	1	Non-easy
17	6	0	0	0	0	0	Easy
18	0	1	1	2	2	0	Non-easy
19	6	0	0	0	0	0	Easy
20	6	0	0	0	0	0	Easy
21	2	0	1	2	1	0	Non-easy
22	6	0	0	0	0	0	Easy
23	2	0	1	0	2	1	Non-easy
24	5	0	0	1	0	0	Easy
25	6	0	0	0	0	0	Easy
Kappa	0.647	-0.001	0.094	0.074	0.022	0.049	

Table 0-4 Table showing overall levels of intra-observer agreement for procedures performed at each level of difficulty (SC1- SC6)

Outcome	Kappa	Z	Prob>Z
SC1	**0.6467**	**12.52**	**0.0000**
SC2	**-0.0007**	**-0.01**	**0.5051**
SC3	**0.0942**	**1.82**	**0.0341**
SC4	**0.0736**	**1.42**	**0.0772**
SC5	**0.0217**	**0.42**	**0.3369**
SC6	**0.0493**	**0.95**	**0.1699**
combined	**0.2904**	**10.19**	**0.0000**

Appendix 4. Meeting abstracts

Poster presentations

National

P174. Interpreting embryo transfer difficulty using a visual analogue scale and its impact on the IVF outcome. *Coats, E; Carden, H; Richardson, A; Hopkisson, J; Raine-Fenning N;* British Fertility Society 5-7th January 2017

International

The impact of difficult embryo transfer on implantation, clinical pregnancy and live birth rates following single blastocyst replacement. *Coats, E; Carden, H, Richardson, A, Zujovic, L Hopkisson, JF, Raine-Fenning, N*; ESHRE 2017

Oral presentations

Regional

University of Nottingham Sue Watson Lecture – 3rd place awarded
The impact of difficult embryo transfer on implantation, clinical pregnancy and live birth rates following the transfer of a single blastocyst

National

Reproductive Medicine Winter Symposium (Saalbach, Austria)
Lawrence Shaw Medal awarded
Embryo transfer: dark art or clinical science