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A glance into the uterus during *in vitro* simulation of embryo transfer

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BACKGROUND: The currently low implantation rate after embryo transfer (ET) is partially attributed to technical aspects, such as catheter type, catheter load, placement of catheter tip and physician skills. METHODS: Mock ET simulations were conducted with a transparent laboratory model of the uterine cavity. The catheter was loaded with alternating air and coloured liquid media. The transfer procedure was recorded by a digital video camcorder for later analysis. Different sequences of air and liquid volumes, as well as liquids of different viscosity were simulated. RESULTS: Injection of liquid with air into the uterus formed an air bubble which blocked the transport of the transferred liquid towards the fundus. The distribution of the transferred matter within the uterine cavity was determined by the composition of the liquid–air sequence and the viscosity ratio between the transferred liquid and the uterine fluid. CONCLUSIONS: It is suggested that the catheter load should contain minimal volumes of air in order to enhance the embryos' chances of reaching the site of implantation. The viscosity of the transferred liquid should be as close as possible to that of the uterine fluid in order to avoid transport of embryos towards the cervix.

Key words: catheter loading/laboratory model/mock embryo transfer/transferred matter

Introduction

The technical protocol of embryo transfer (ET) has not been changed since it was first introduced three decades ago (Edwards *et al*., 1984). Typically, the catheter is positioned within the uterine cavity with its tip 5–15 mm proximal to the fundus, whereupon its load is carefully injected and the catheter is then extracted. Retrospective studies of IVF described relatively high success rates (>90%) of ova fertilization in the laboratory compared with a low rate of successful outcome (<25%). Mechanical factors, such as uterine contractions, catheter type, the method of loading the catheter, the placement of the catheter tip and physician skills, have been proposed to explain some of the disparity between the embryonic development and pregnancy rates (Kovacs, 1999; Schoolcraft *et al*., 2001).

The embryos are transferred into the uterine cavity within a liquid medium following a variety of protocols that vary between clinics. The liquid medium containing the embryos is placed near the distal end of the catheter and separated by air buffer zones to avoid spillage of the embryos (Kan *et al*., 1999). The rest of the catheter and the syringe are filled either with air (i.e. volumes ranging between 30 and 100 µl) or with a combination of air and liquid media. Replacing the air within the transferred matter with a similar volume of liquid increased the rates of implantation and pregnancy (Meldrum *et al*., 1987). It has been advised that while transfer volumes >60 µl may result in expulsion of the embryos through the cervix into the vagina (Poindexter *et al*., 1986), volumes of 30 µl may avoid any occurrence of embryo expulsion from the uterus (Mansour *et al*., 1994). Recently, it was also suggested that transferred volumes <10 µl as well as the presence of air bubbles may have a negative influence on the implantation rate (Ebner *et al*., 2001). In order to avoid ectopic pregnancies, which were recognized as a potential risk of IVF, it was recommended to transfer volumes <30 µl (Yovich *et al*., 1985; Nazari *et al*., 1993; Marcus and Brinsden, 1995; Baba *et al*., 2000; Ebner *et al*, 2001; Bilalis *et al*., 2002).

Intrauterine embryo transport after ET is also determined by the pattern of mixing between the transferred media and the uterine fluid, which have different viscosities. While the transferred liquid has a viscosity similar to that of water, the uterine fluid is expected to have a relatively higher viscosity, but information for humans is not available. This assumption may be supported by recent research of the rheological characterization of uterine fluid of mares, which revealed that the gel-like uterine fluid has a viscosity of ~1000 centipoise

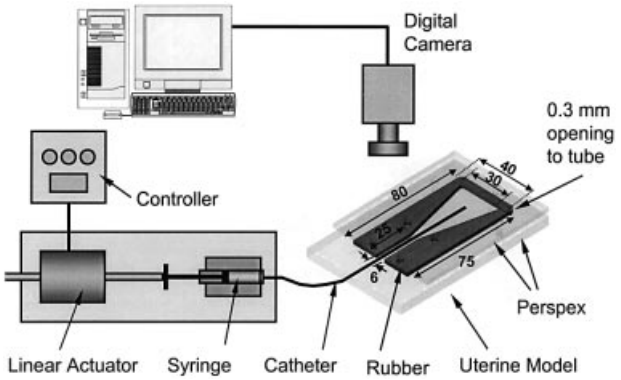

Figure 1. Schematic description of the experimental set-up.

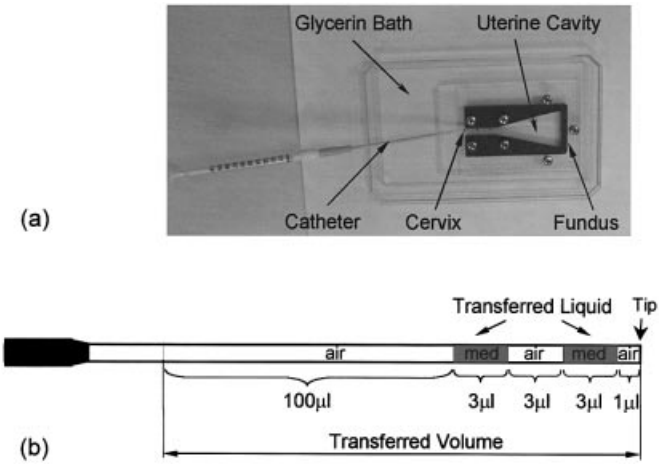

Figure 2. (**a**) A uterine model within a glycerin bath. (**b**) The catheter load with a sequence of air and liquid volumes.

(please note that in SI units 1 $Ns/m^2 = 10^{-3}$ centipoise) that decreases with increased shear rate or pressure (Allen *et al*., 2002). Another study with human cervical mucus revealed minimal values of 1500–2500 centipoise during ovulation and ~10^5 centipoise during the secretory phase (Karni *et al*., 1971). Nevertheless, clinical experiments with a transferred medium of larger viscosities (e.g. 100 times the viscosity of water) did not show an increase in implantation rates (Menezo *et al*., 1989).

Once the embryo enters the uterus via normal or assisted reproduction, it is conveyed with the intrauterine fluid motions which are induced and controlled by myometrial contractions for 2–3 days until implantation occurs (Eytan and Elad, 1999). Clinical studies of ultrasound-guided ET procedures revealed that the introduction of the catheter into the uterine cavity interfered with normal uterine peristalsis and induced irregular contractions that may push the embryo away from the implantation site (Woolcott and Stanger, 1997; Fanchin *et al*., 1998; Lesny *et al*., 1998; Ghazzawi *et al*., 1999; Ijland *et al*., 1999). It has been speculated that withdrawal of the catheter results in movements of the air bubble towards the cervix, which could expel the embryos from the uterus (Poindexter *et al*., 1986; Lesny *et al*., 1998; Schoolcraft *et al*., 2001). In this study, we developed an ET model to investigate the dispersion

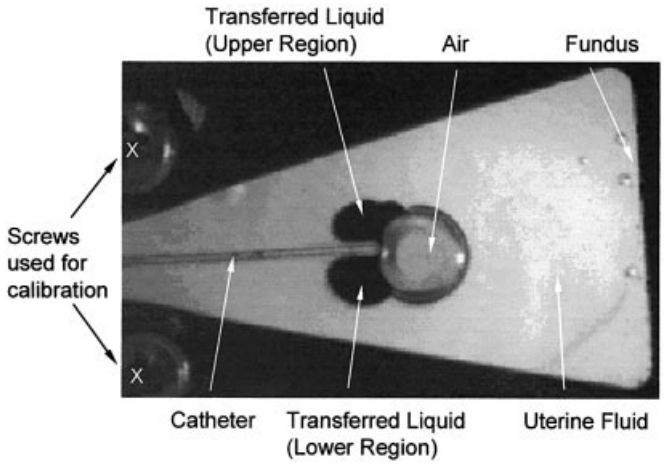

Figure 3. A typical result of a mock ET experiment in the uterine model.

of the transferred volume within a mock uterine cavity for various protocols of ET in order to study the post-ET positioning of the transferred embryos.

Materials and methods

Experimental apparatus

Mock ET simulations were conducted in a laboratory model of the uterine cavity. The experimental set-up that mimicked the ET procedures in the uterus consisted of a rigid uterine model, a linear actuator and a digital video camcorder (schematically described in Figure 1). The rigid uterine model was composed of two rectangular Perspex boards separated by a 2.5 mm thick rubber pad which had the geometry of the inner cavity of the uterus in the frontal cross-section with two 0.3 mm openings for the Fallopian tubes (Figure 2a). The uterine model was positioned in a bath filled with glycerin, which also filled the cavity in order to mimic the viscose uterine fluid. We chose to use glycerin since its viscosity is 1880 centipoise which is believed to be of the same order as that of the uterine fluid (Allen *et al*., 2002). The Cook catheter (Cook Australia, Australia) was inserted through the narrow opening which represented the cervix of the uterine model. The catheter tip was positioned 2.5 cm from the fundus in all the simulations. The transferred liquid was either saline (1 centipoise), glycerin (1880 centipoise) or P-1 medium (Irvine Scientific, CA) (2 centipoise). The transferred liquid was coloured by 10 mg of bromophenol blue dissolved in 10 ml of 10 mM Tris pH 8. The delivery speed of the transferred matter was set to 20 μl/s by adjustment of the linear actuator. The transfer procedure was recorded by the video camcorder for ~10–15 s, at a rate of 10 images/s.

Experimental protocol

The mock ET simulations were based on protocols similar to the real ET procedure as carried out in our IVF Unit. The catheter was loaded with a series of alternate air and transferred liquid (e.g. saline, P-1 or glycerin) volumes described from the catheter tip (Figure 2b). The following cases were simulated: (i) case I (control), 1 μl of air, 3 μl of saline (which usually contains the embryos), 3 μl of air, 3 μl of saline and 100 μl of air; (ii) case II, 1 μl of air, 3 μl of saline, 3 μl of air, 3 μl of saline and 200 μl of air; (iii) case III, 1 μl of air, 3 μl of P-1, 3 μl of air, 3 μl of P-1 and 100 μl of air; (iv) case IV, 1 μl of air, 3 μl of glycerin, 3 μl of air, 3 μl of glycerin and 100 μl of air; and (v) case V, 1 μl of air, 3 μl of saline,1 μl of air and 100 μl of saline.

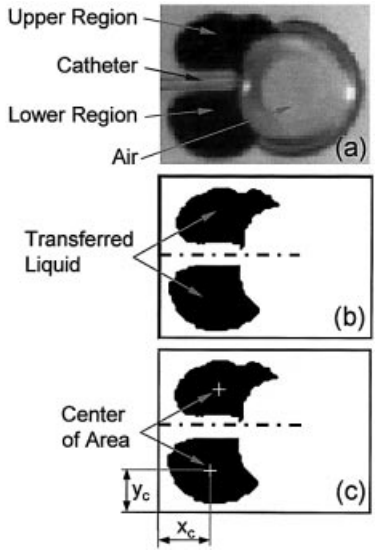

Figure 4. Image processing of a mock ET. (**a**) An instantaneous image during the injection process; (**b**) a binary image of the image in (a) in order to identify the transferred liquid; (**c**) the centre of the area for the upper and lower regions.

Each of these cases was simulated three times to ensure reproducibility of the results. The following terminology will be used: 'transferred volume' represents the overall transferred volume of liquid with air, and 'transferred liquid' represents the overall transferred liquid without air.

Analysis of the dynamic expansion of the transferred matter

The potential intrauterine positioning of the embryos during the ET process was analysed from the tracing of the transferred coloured liquid which contains the embryos in a real ET procedure. The recorded digital images of the mock ET simulations revealed a typical distribution of the coloured transferred liquid (Figure 3). For each experiment, we analysed consecutive images, which represent a rate of 10 images/s. First, the image size was calibrated by measuring the distance between the screws that hold the Perspex boards at both sides of the cervix in the uterine model (Figures 2a and 3). Then, the region of interest was determined from the last image in the sequence which included the largest spatial dispersion of the transferred matter (Figure 4a). The same region of interest was extracted from all the images in order to reduce the processing time of the dynamic analysis.

The interest of this research was to study post-ET positioning of the transferred embryos, therefore, one should analyse the distribution of the transferred liquid, where the embryos are likely to be. The instantaneous area occupied by the spreading transferred liquid was detected in each image by the purple colour of the liquid. Observation of a typical image shows that the transferred liquid had spread into two regions on both sides of the catheter, which were defined as 'upper' and 'lower' (Figure 3). Each region of the distended transferred liquid was analysed separately by edge detection techniques (Figure 4). The original image of 24-bit RGB (red, green, blue) in the Bitmap format (Figure 4a) was converted into a binary file of black and white (Figure 4b), where black denoted staining of the transferred liquid. The instantaneous area of the stained liquid was calculated by summing up all the black pixels in each region. The dynamic expansion of the coloured liquid (i.e. black area in Figure 4b) may be described by the instantaneous size of its area and the position of its centre of mass in consecutive images. The centre of a two-dimensional shape is the average position of all the pixels that make up a particular shape. Accordingly, the centre of an area of N pixels is defined by

$$x_c = \frac{\sum_{i-1}^{N} A_i x_i}{\sum_{i-1}^{N} A_i}, \qquad y_c = \frac{\sum_{i-1}^{N} A_i y_i}{\sum_{i-1}^{N} A_i} \tag{1}$$

where A_i, x_i and y_i are the black area and coordinates of each pixel, respectively, and x_c and y_c are the coordinates of the centre of the area (Figure 4c). Since all the pixels of a digital image have the same area, Equation 1 may be reduced to

$$x_c = \frac{\sum_{i-1}^{N} x_i}{N}, \qquad y_c = \frac{\sum_{i-1}^{N} y_i}{N} \tag{2}$$

The centre of the area of a two-dimensional shape is equivalent to the centre of mass of a three-dimensional object. The coordinates of the centre of the area were calculated for each region in the image (Figure 4c).

Results

Mock ET simulations were conducted for five protocols of the catheter load, and the dynamic dispersion of the transferred volume within the uterine cavity during the injection phase was analysed. The total air volume transferred into the uterine model formed a single air bubble which was attached to the catheter tip, while the transferred liquid (i.e. the dark marks) spread out on both sides of the catheters (Figure 3). The final shape of the transferred volume within the uterus was composed of the two 3 μl liquid volumes and a large (i.e. 104 μl) air bubble (Figure 5a). As the air came off the catheter tip (immediately after the onset of injection), it formed two small volumes of air (1 and 3 μl), which joined to become one large air bubble as the injection proceeded.

The transferred matter was injected forward towards the fundus. As the air came off the catheter, the air bubble was positioned at the catheter tip, blocking the passage of the transferred liquid forward (Figures 5–7). As a consequence, the transferred liquid spread backward towards the cervix, as soon as the first liquid volume (which in the real ET contains the embryos) came off the catheter. The dynamic expansion of the transferred liquid was described in terms of the increase in its area with time and the movement of the centre of the area (Figure 5). For the control case (I), the centre of the upper and lower areas moved ~2.5 mm from the vicinity of the catheter tip towards the cervix (Figure 5b). The coloured liquid, however, expanded for a length of ~10 mm, and its edge was located 8 mm from the catheter tip towards the cervix. The area of the transferred liquid increased linearly from the first second after the injection (Figure 5c).

Increasing the amount of air from 100 to 200 μl (case II) resulted in a larger air bubble (Figure 6a) which further inhibited the dispersion of transferred liquid. As a consequence, the two regions into which the transferred liquid spread

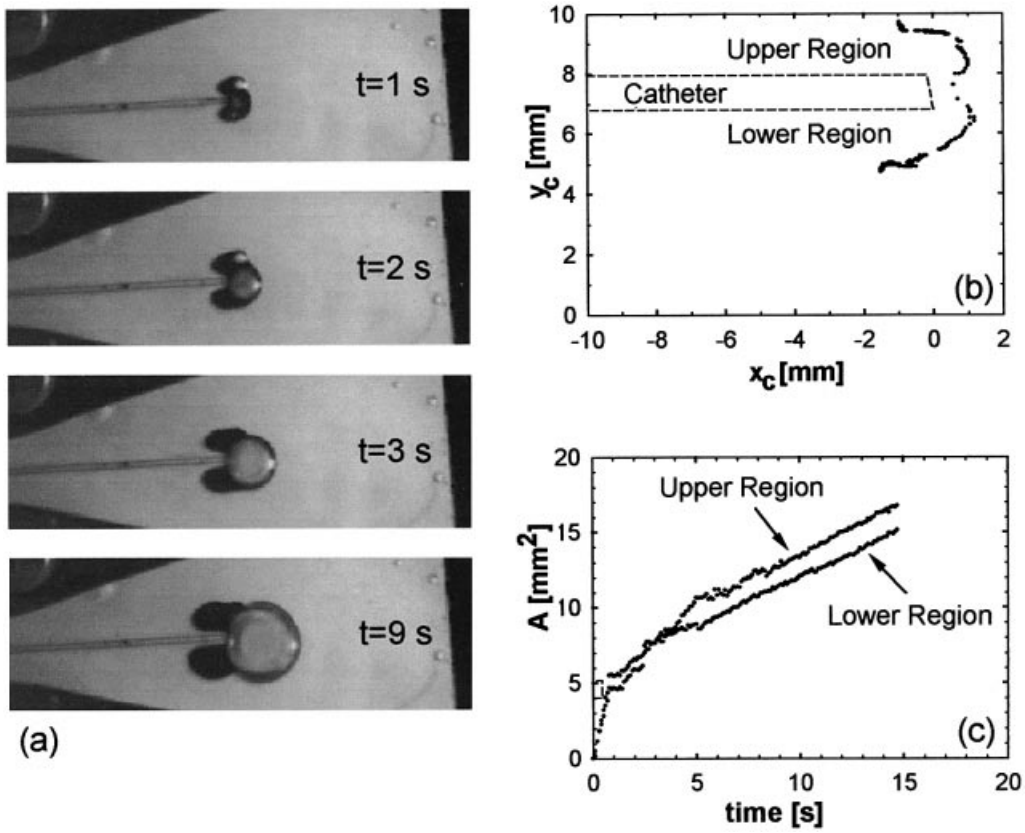

Figure 5. Case I: injection of air and saline into the uterine model. (**a**) Instantaneous images during mock ET; (**b**) movements of the centre of the area of the transferred liquid (zero denotes the tip of the catheter); (**c**) the time-dependent area of the transferred liquid (upper and lower regions).

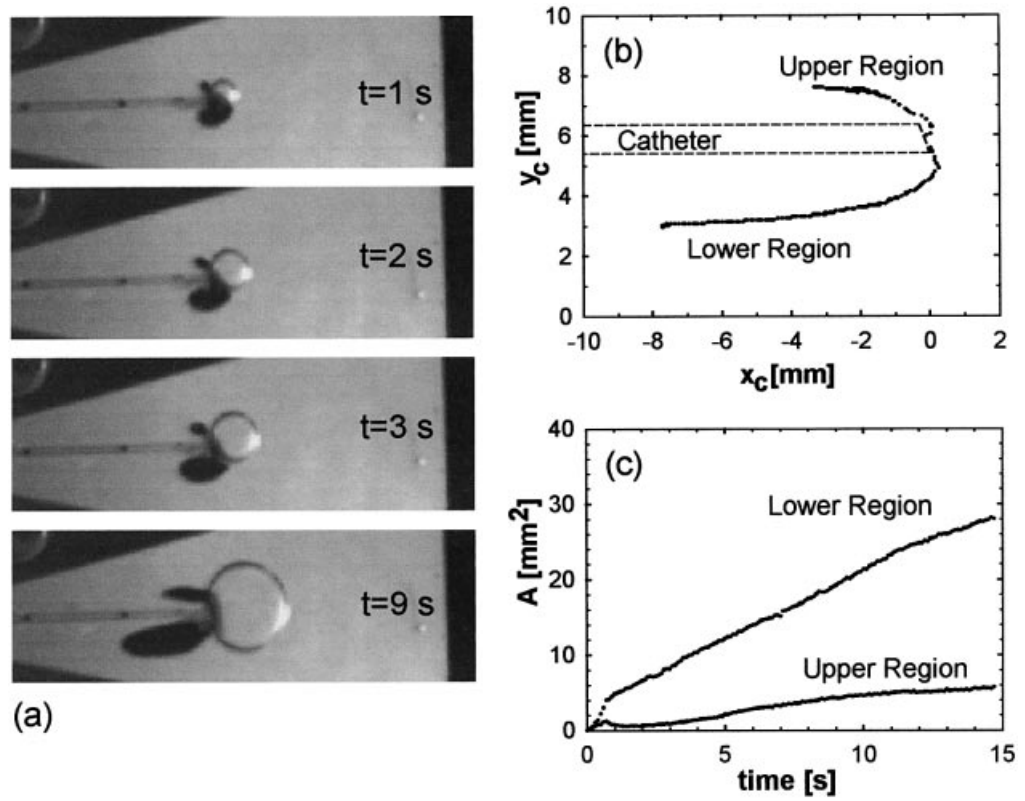

Figure 6. Case II: injection of a greater volume of air and saline than in case I into the uterine model. (**a**) Instantaneous images during mock ET; (**b**) movements of the centre of the area of the transferred liquid (zero denotes the tip of the catheter); (**c**) the time-dependent area of the transferred liquid (upper and lower regions).

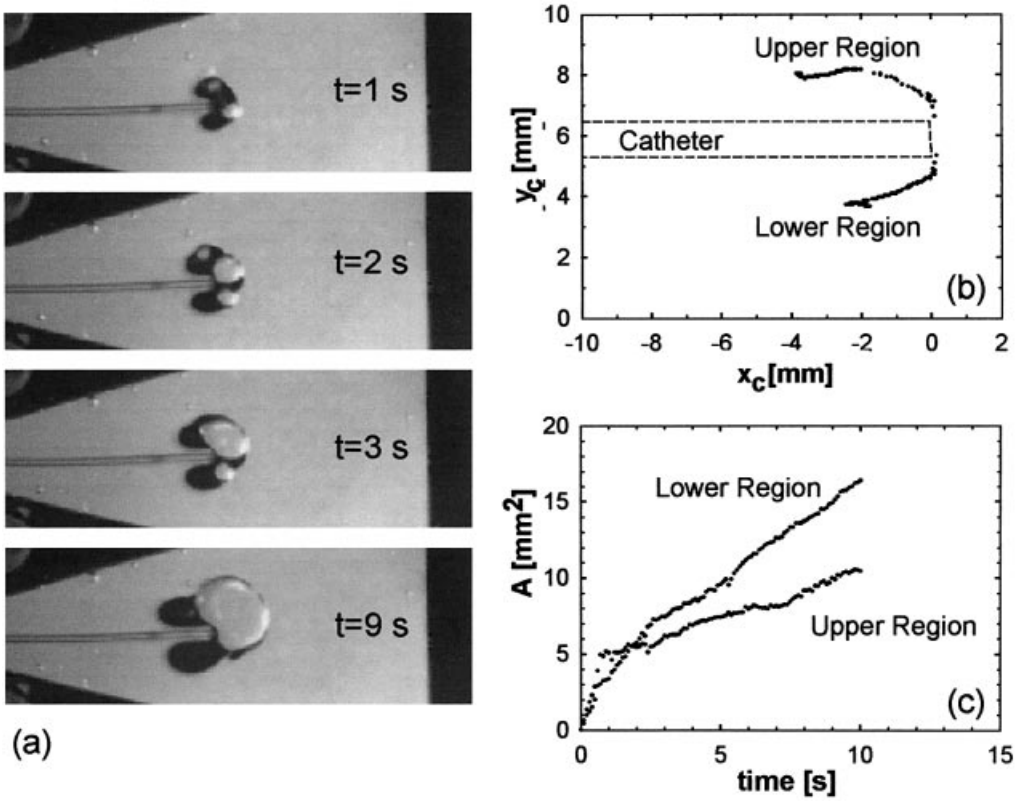

Figure 7. Case III: injection of air and P-1 into the uterine model. (**a**) Instantaneous images during mock ET; (**b**) movements of the centre of the area of the transferred liquid (zero denotes the tip of the catheter); (**c**) the time-dependent area of the transferred liquid (upper and lower regions).

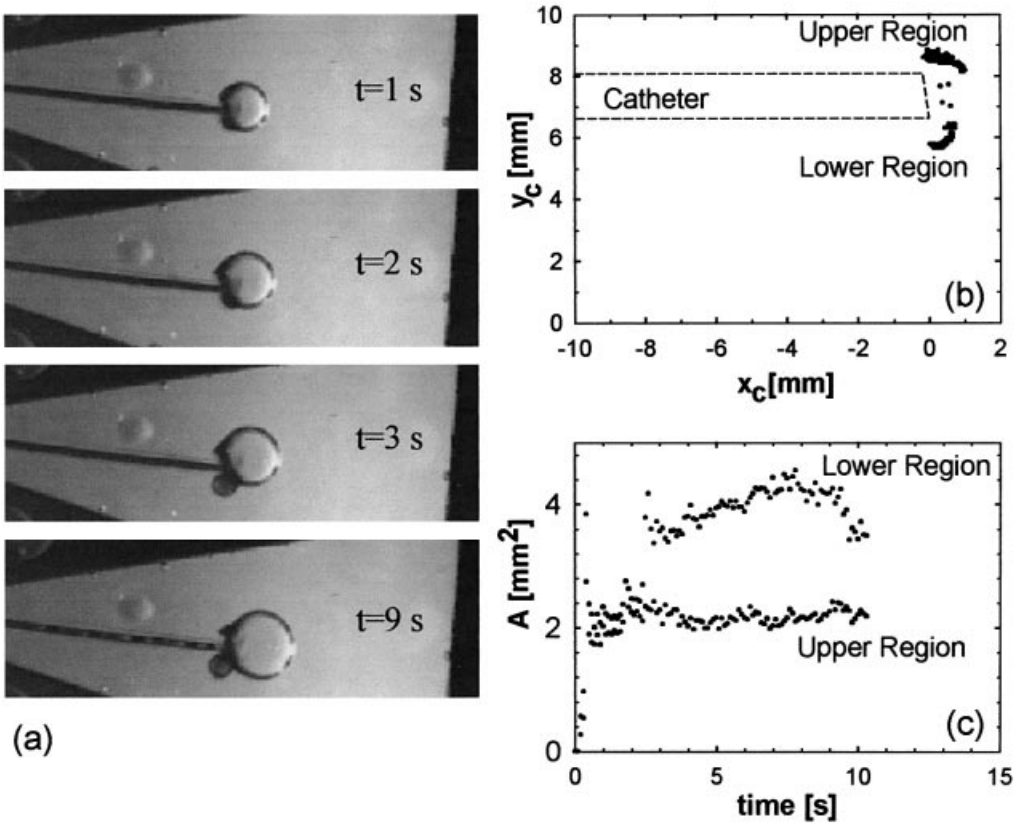

Figure 8. Case IV: injection of air and glycerin into the uterine model. (**a**) Instantaneous images during mock ET; (**b**) movements of the centre of the area of the transferred liquid (zero denotes the tip of the catheter); (**c**) the time-dependent area of the transferred liquid (upper and lower regions).

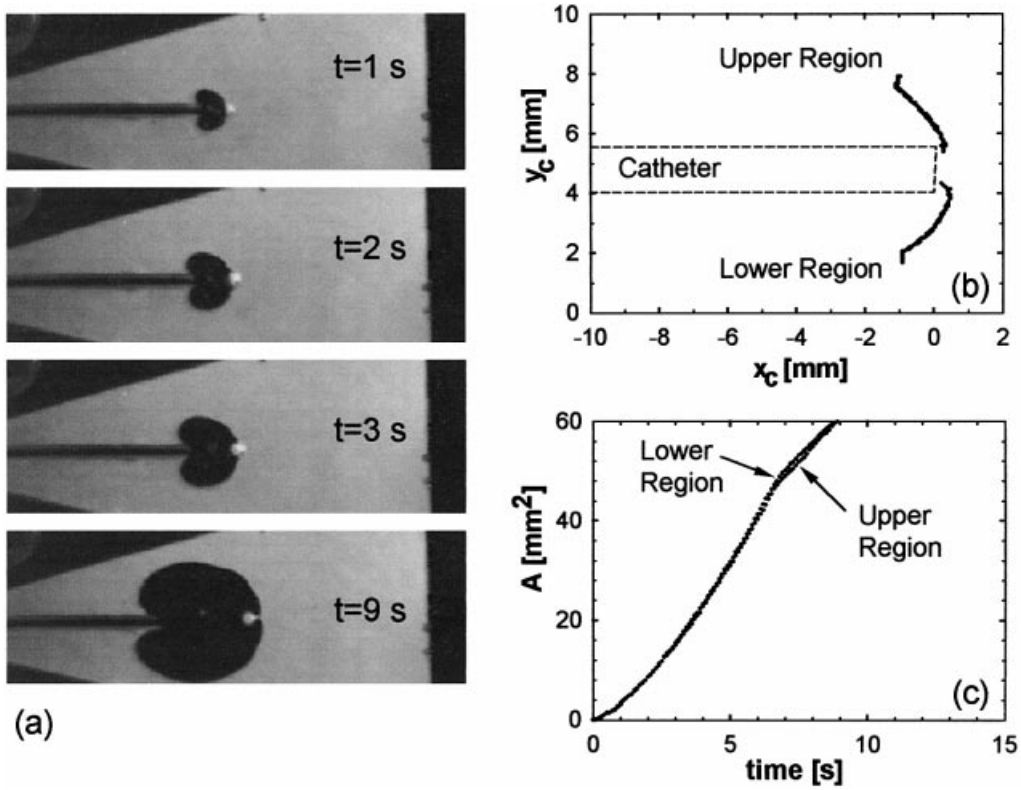

Figure 9. Case V: injection of air and saline into the uterine model in which some of the air volume is replaced by liquid. (**a**) Instantaneous images during mock ET; (**b**) movements of the centre of the area of the transferred liquid (zero denotes the tip of the catheter); (**c**) the time-dependent area of the transferred liquid (upper and lower regions).

were not symmetric as had been the case for the 100 µl air volume (Figure 5a). The centre of the area moved 3 and 8 mm for the upper and lower regions, respectively (Figure 6b). The larger axes of the upper and lower regions were 8 and 16 mm, respectively, with their apexes at 6 and 13 mm from the catheter tip in a direction towards the cervix. The area of the regions increased linearly from the onset of injection but at different rates (Figure 6c).

When the transferred liquid was P-1 (i.e. case III), whose viscosity is twice that of saline, all the air volume appeared as separated bubbles at the beginning of the injection which merged later into one large bubble (Figure 7a). The final structure of the coloured liquid is similar to the pattern obtained in case I (Figure 5). The centre of the area moved 3–4 mm from the catheter tip towards the cervix. The transferred liquid spread for ~10 mm in length, 7.5 mm from the tip towards the fundus (Figure 7b). The area of the liquid regions increased linearly from the first second (Figure 7c).

When the transferred liquid was identical to that within the uterine model (i.e. glycerin, case IV), the final structure of the transferred matter within the cavity was an air bubble with the transferred liquid spread around its circumference (Figure 8a). In this case, the transferred liquid remained at the vicinity of the catheter tip, its area remained fairly constant (Figure 8c) and the centre of the area revealed a movement of 1 mm towards the fundus (Figure 8b). The dispersion of the transferred liquid was limited to the circumference of the air bubble.

Replacement of the 100 µl volume of air (Figure 2b) with a 100 µl volume of liquid (i.e. case V) produced a different pattern, with a small air bubble (3 µl) appearing at the front (Figure 9a). At the beginning of the injection, the coloured liquid had a shape similar to that in case I. As the injection proceeded, however, the transferred liquid took the shape of an incomplete ellipse which covered the catheter tip (Figure 9). A single region was usually present, but it was divided into upper and lower regions for the purposes of comparison with the other ones (Figure 4d). The motion of the centre of the area of the upper and lower regions revealed movements towards the catheter side, with a <2 mm displacement, from the catheter tip towards the cervix (Figure 9b). The liquid spread for a length of 13 mm, up to a distance of 8 mm from the tip towards the cervix. The increasing rate of the transferred liquid area was linear and larger than in all the other cases.

Discussion

The present study is concerned with the dispersion of the transferred volume in the uterine cavity which may serve as a predictor of possible locations of the embryo after an ET procedure. Although the protocols of the catheter load vary between different IVF units, there are similarities in the use of small transferred volumes which are composed of a sequence of air and liquid contents (Meldrum *et al*., 1987; Bilalis *et al*., 2002). In our IVF unit, the air and liquid content of the catheter load ensures that the embryos will not stick to the catheter wall

during the injection (Figure 2b). The 1 µl volume of air at the catheter tip prevents spillage of the medium containing the embryos (Ebner *et al*., 2001). The 3 µl gap of air between the medium and the embryo and the next bolus of medium prevents the transport of the embryos within the catheter. The liquid that does not contain the embryos and the 100 µl volume of air are added in order to flush the catheter during the injection in case one of the embryos sticks to the catheter's wall. The direct effect of injecting these contents into the uterine cavity on the transport of the embryo within the cavity has not yet been defined.

The ultimate goal of ET is to bring the embryo close to the fundal wall in order to provide optimal conditions for implantation in the uterine wall. The present results revealed that an air bubble had been developed and remained near the catheter tip while the transferred liquid was transported towards the cervix. The coloured liquid which represents potential locations of the embryos could not propagate forward because of that air bubble and, thus, it expanded backward towards the cervix. The liquid volume which includes the embryos in a real ET procedure was very small (e.g. 3 µl); however, the two liquid volumes merged from the onset of injection, thus the embryos could be found anywhere in the area where the transferred volume had been dispersed. The coloured liquid sometimes demonstrated an asymmetric geometry, a possible result of the catheter being slightly curved. In these cases, the air bubble did not coincide with the axis of the catheter and, consequently, the injected liquid was forced into non-identical regions at the side. Asymmetric marks were observed in all simulations of case II when the air bubble had a volume of 200 µl (Figure 6) since it did not remain along the catheter's axis.

The spreading pattern of the transferred matter within the uterine cavity was highly dependent on the volume of air and the viscosity of the liquid within both the uterine cavity and the catheter. The air bubble is stable due to the surface tension that results from the viscosity of the liquids. Because of glycerin's high viscosity (e.g. 1880 centipoise), the air bubble remained near the tip of the catheter. In our simulations, the viscosity of the injected saline (e.g. 1 centipoise) was much lower than that of glycerin, thus the injected liquid could not push the air bubble or the glycerin forward and the liquid was pushed backwards (Figures 5–7). When the injected liquid had the same viscosity as the liquid within the cavity (as in case IV), the transferred liquid accumulated around the air bubble (Figure 8). Since the viscosity of P-1 is about twice that of saline, the transferred liquid spread less from the catheter tip, ~7.5 mm towards the cervix compared with 8 mm in the case of saline. The movement of the centre of the mass of the coloured liquid was larger in the case of P-1 (e.g. 3–4 mm compared with 2.5 mm in saline) since it spread towards the sides, giving rise to wider marks. The spreading pattern of the transferred liquid indicates that the viscosity of the transferred liquid should be as close as possible to the uterine fluid in order to avoid the dispersion of the transferred liquid with the embryos towards the cervix.

Menezo *et al*. (1989) reported that increasing the viscosity of the transferred matter from 2 to 120 mPa/s did not improve the outcome of ET. It may be that the viscosities used by Menezo *et al*. (1989) were too low compared with the uterine fluid and, thus, the distribution of the transferred volume within the uterine cavity was similar to the shape of cases I and III (Figures 5 and 7), yielding a similar outcome of ET. Based on the present simulations, it may well be that investigation of the effect of the viscosity of the transferred liquid on the implantation rate requires much higher viscosities.

The volume of 100 µl of air was responsible for the formation of one air bubble (Figure 5). When the transferred air was substituted by liquid (i.e. saline in case V), the transferred liquid remained in the vicinity of the tip of the catheter (Figure 9), which will bring the embryos closer to the fundus in a real ET. A better ET outcome is achieved when the catheter is positioned 5–15 mm from the fundus, a position that ensures that the embryos reach the fundus to implant there (Lesny *et al*., 1998; Schoolcraft *et al*., 2001; Mansour and Aboulghar, 2002). Consequencely, one may increase the liquid's volume in the transferred matter and probably reduce the air volume which is responsible for the embryos being spread towards the cervix. Mansour *et al*. (1994) showed that the presence of two 5 µl air bubbles on both sides of 15 µl of transferred liquid did not induce the expulsion of the transferred matter through the cervix as long as the total volume did not increase. Our observations support this result since in none of the simulations was the transferred liquid ever expelled from the uterine model.

When all the transferred volume is composed of liquid, however, the embryos may be distributed throughout a larger region (Figure 9). On the other hand, it may be beneficial to use air in the transferred liquid since it is more quickly absorbed by the uterine tissue than the liquid. Nevertheless, our model could not simulate the rate of absorbance or disappearance of the air. Using a larger volume of air (e.g. 200 µl) resulted in a larger bubble which almost doubled the distance the embryos moved towards the cervix (Figure 6), a finding which supports the reports that implantation and pregnancy rates increased when the amount of air and total transferred volume were reduced (Meldrum *et al*., 1987; Ebner *et al*., 2001). Accordingly, the transferred volumes of air should be small enough to avoid the transport of the embryos into the cervix. It should be noted that a small volume of air at the edge of the catheter load (Figure 2b) was required to avoid liquid spillage while the catheter was inserted into the uterine model (Ebner *et al*., 2001).

The transferred matter was injected at a constant velocity in all the experimental protocols of this study, and the area of the coloured liquid increased linearly, as demonstrated in Figures 5c, 6c and 7c. In contrast, when the injected liquid was glycerin (i.e. the viscosity was similar to that of the uterine fluid), the total area of the dispersed liquid remained fairly constant at ~6 mm^2 (Figure 8c). The area of the transferred liquid was smaller than in cases I–III since the transferred liquid distributed around the air bubble and lost its dark colour and, as a consequence, could not be detected by the algorithm of this study.

The uterine model in this work was rigid and, therefore, could not simulate uterine contractions, which were suggested

as the mechanism responsible for pushing the embryo towards the site of implantation. Mathematical models demonstrated that normal uterine contractions induce intrauterine fluid flow which results in embryo transport (Eytan and Elad, 1999). However, increased uterine contractions, which may occur during insertion of the catheter, have been suggested to be responsible for reduced rates of implantation (Fanchin *et al*., 1998). In a recent computer simulation of ET within a channel with oscillating walls, we demonstrated that the injection velocity as well as uterine wall peristalsis significantly affect the trajectories of the embryos (Yaniv *et al*., 2003). In this study, the speed of injection of the transferred matter was limited to a single velocity of 20 μl/s due to system limitations. The effect of the speed of injection on the distribution of the transferred matter within the uterine cavity should be investigated further.

In our uterine model, the final shape of the transferred matter was attached to the tip of the catheter, which may not be the case in a real ET. The presence of uterine peristalsis may transport the transferred matter forward towards the fundus. These contractions may also separate the transferred liquid and the air or mix the transferred matter with the uterine fluid in a way that helps the embryo reach the fundal area during the time window for implantation. Baba *et al*. (2000) reported that embryos were implanted at the area where they were initially placed, which may imply that transferred embryos have not been transported away by uterine contractions. In addition, clinical experience has shown that withdrawal of the catheter may create a negative pressure which may pull out the embryos from the uterine cavity (Mansour and Aboulghar, 2002). This observation may be supported by the present findings that the transferred volume remained in the vicinity of the catheter tip. A fast withdrawal of the catheter may pull out the embryos through the cervix and the vagina.

In summary, ET is a mechanical procedure, in which two materials (e.g. liquid and air) are transferred in sequence into the uterine cavity which is filled with a more viscous fluid. This study showed that the composition of the transferred volume determines the spreading pattern of the transferred matter within the uterine cavity and, accordingly, controls the transport of the embryos. A better understanding of the transport of embryos within the transferred matter, the ultimate viscosity and absorbance of the transferred matter in the uterine cavity will improve the protocols for ET procedures and increase the success rates of embryo implantation and pregnancies.

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