Journal of Assisted Reproduction and Genetics, Vol. 19, No. 8, August 2002 (© 2002)

Clinical Assisted Reproduction

Objective Assessments of Temperature Maintenance Using In Vitro Culture Techniques[1]

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Submitted January 10, 2002; accepted March 8, 2002

***Purpose*:** To assess the ability of various facets of embryo culture (microscope stage warmers, volumes of culture media, culture vessel lids, and type of culture incubator) to maintain a constant temperature in vitro.
***Methods*:** Ability to maintain 37.0°C in the microenvironment of gametes was recorded by digital thermocouple in the chosen facets of in vitro culture.
***Results*:** Stage warmers are highly variable in their ability to maintain the set temperature (range 33.8°C–37.0°C after 60 s). Temperature loss in culture media is both volume and vessel dependent, and the direct heat transfer culture incubator (MINC) has superior temperature maintenance compared with a large volume air convection incubator (FORMA), where temperature regain from 35.0°C to 37.0°C took 5.5 min compared to >20 min.
***Conclusions*:** There are large measurable differences in the ability to maintain set temperature that depend on the stage warmer used, volume of media, use of vessel lids, and the type of incubator chosen for IVF culture.

KEY WORDS: Culture volume; incubators; oil overlay; stage warmers; temperature.

INTRODUCTION

Variation from optimal culture temperature has been shown to affect cleavage and blastocyst formation in bovine embryos (1), whether due to short-term or prolonged temperature shock (2). It has been suggested that human microtubule spindles are thermosensitive (3) and that optimal temperature for human oocytes should be 37°C as changes in temperature can irreversibly affect spindle integrity (4). Interestingly, it has been suggested that the correct temperature of the follicular fluid is actually lower than both the ovarian stroma and body temperature, and that the temperature of the follicular fluid rises as ovulation approaches (5). These authors suggest that there may be a period of reduced follicular temperature necessary to achieve resumption of meiosis. After this, the temperature regulation at 37°C may be critical to maintain spindle integrity (4). This temperature fluctuation, which can easily occur in routine assisted reproductive technologies (ART), may result in major abnormalities of chromosomal distribution. Suboptimal embryo development can be displayed as fragmented embryos undergoing programmed cell death or apoptosis, or as embryos with delayed cellular division (6). Apoptosis can also lead to preimplantation failure (6), and may occur early in human embryos conceived in vitro. This could represent a response to suboptimal culture conditions (7), with levels of cell death in embryos cultured in vitro greater than in embryos in vivo (8). Alterations in culture conditions and/or hormonal stimulation have also been

[1] The authors did not receive any funding or inducements from any manufacturer mentioned within this paper, or have any vested interest in any particular equipment.
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suggested to induce chromosomal abnormalities and may partly explain differences in pregnancy rates between in vitro fertilization centres (9). It was recently shown that temperature fluctuations depend on the size of the dish used and the volume of oil used as an overlay of culture droplets (10); however, there was no mention of the type of stage warmer used or the speed of the temperature loss. Despite the culture of human embryos for over two decades the literature is still incomplete with respect to maintenance of constant temperature when using different types of equipment and environments. The aim of this study was therefore to assess the ability of maintaining a set temperature of 37.0°C measured at the culture surface with respect to several microscope stage warmers and incubators. The volume of culture media, volume of oil overlays, and the use of culture dish lids will also be assessed.

MATERIALS AND METHODS

Temperature was measured using a digital thermometer (S506, LEC Instruments, Australia) using a thermocouple (Type-K, LEC Instruments, Australia) with the combination calibrated by a National Association of Testing Authority accredited laboratory, between 0°C and 37°C with an accuracy of ±0.1°C. Stage warmer temperatures were obtained from both a controlled environment inside a Class II cabinet (BH2000 Class II, Clyde-Apac, Australia) and on inverted microscope stages used for microinjection procedures. Culture dishes for Experiments A–D involved plastic 4-well Nunc dishes (Cat # 76740, Medos, Australia). A shallow-sided plastic ICSI dish (Falcon 1006, Bacto, Australia) was used for the inverted microscope temperature readings in Experiment A. For readings with the culture dish lid on, the 4-well Nunc dish had a 1.5-mm hole drilled in the lid in the centre of a culture well that allowed the thermocouple to be placed through the hole and taped in place. When the lid of the 4-well Nunc dish was not required, the thermocouple was held in place by taping to the dish itself. Readings taken using the Falcon 1006 dishes had the thermocouple stabilized by being taped to the condenser turret of the inverted microscope.

The copper thermocouple wire had the distal end coiled around fine forceps to maximize the area of the thermocouple in the media volume being tested. The coil was then flattened and bent at 90° so the coiled thermocouple lay flat along the culture surface and the excess wire ran upwards to be taped securely in place to avoid loss of measurement parameters if the dish was moved. This ensured that the temperature readings were taken at the culture surface, with as much of the thermocouple wire inside the fluid as possible.

Experiment A. Effect of Microscope Stage Warmers

The two stage warmers used in a controlled airflow cabinet were a solid glass stage warmer (MP30DM, Kitazato, Japan), which has the whole surface as a warmed glass stage, and a solid matte-black stage warmer surface (LEC978, LEC Instruments, Australia).

Two inverted microscope stage warmer systems used for microinjection were also tested. One was a matte-black stage warmer surface (LEC916, LEC Instruments, Australia) with a 15 mm diameter hole removed in the centre to facilitate sample viewing during microinjection. This type of warmer allowed temperature readings to be obtained from two locations—over the viewing hole and over the solid part of the warmed stage ring. This stage warmer was compared with a full perspex environmental chamber and heater blower (ITC32, Nikon, Japan) complete with a heated glass stage ring (MATS505R30, Tokai Hit, Japan).

The stage warmer temperatures were set according to the manufacturers' instruction books to maintain a desired temperature of 37°C. The MP30DM was set at 37°C, and the LEC978 and the LEC916 were set at 38°C. The calibration temperature for the environmental chamber (ITC32) was reduced to 35.5°C as the manufacturer's set point proved very inconsistent, and was consistently too high, giving chamber temperatures in excess of 38.0°C, a temperature high enough to denature enzymes and proteins within the embryo. When the set temperature was reduced to 35.5°C, the chamber temperature never exceeded 37°C. Temperature for the stage warmers was recorded every 5 s up to 1 min; every 10 s up to 5 min; then every minute.

Experiment B. Effect of Media and Oil Volume

The culture volumes assessed were a 50 μL droplet of media (Cat # 1026, SAGE BioPharma, USA) with a 1.0 mL oil overlay (Cat # 4008, SAGE BioPharma, USA), and the inverse situation with 1.0 mL of media and a 0.1 mL oil overlay. Temperature for the culture volumes was recorded every 5 s up to 1 min; every 10 s up to 5 min; then every minute.

Experiment C. Effect of Culture Vessel Lid and Media Volume

The most accurate stage warmer was used to assess the effect of including or excluding the lid of the 4-well Nunc dish on culture media temperatures. Temperature was recorded in both the media volumes from Experiment B. Temperature was recorded every 5 s up to 1 min; every 10 s up to 5 min; then every minute.

Experiment D. Effect of Culture Incubator

The two incubators assessed had different modes of action to disperse their heat. The large volume incubator (FORMA #3194, Selby, Australia) works by transmitting heat from the water jacket to the internal chamber air, which warms the culture dish and then the culture media. This incubator has one internal glass door and one external heated door. The MINC1000 (COOK, Australia) works by direct heat transfer from a recessed incubator heated surface straight to the surface of the culture dish containing the culture media. This incubator has one heated door covering each chamber.

To differentiate between actively heated culture surfaces (MINC) and convection heated culture shelves (FORMA), a third device has been assessed. This is an INCUPLATE (CIP-1000, COOK Australia), which is a solid metal recessed insert for the large volume incubators which acts as a direct transfer of retained heat absorbed from the incubator chamber. The temperature of the dishes was reduced to 35.0°C to simulate embryo assessment on an ineffective stage warmer, and were then placed into the incubator to determine the elapsed time to return the media to 37.0°C. Culture volumes assessed were the same as for Experiment B. The chamber doors of the incubator being assessed were opened for no longer than 5 s; this was just long enough to carefully place the culture dish within the incubator. Temperature recordings were taken every 20 s up to 5 min; then every 30 s up to 10 min; then every minute up to 20 min.

RESULTS

Experiment A. Effect of Microscope Stage Warmers

The temperature maintenance from the four different microscope stage warmers can be seen in Fig. 1. A reference line is also included showing temperature decrease when a culture dish is placed on an unheated work surface. Starting temperatures were 37.0 ± 0.1°C.

It can be seen that the temperature drop on the inverted microscope stage warmer (LEC 916) was 4.8°C over 2 min when measured over the viewing hole, but the decrease in the same media droplet measured over the solid pad component of the warmer was 2.3°C. In comparison, the environmental chamber dropped by a corresponding 0.8°C in 2 min. This chamber then took another 90 s to steadily drop to the set temperature of 35.5°C, which it then maintained.

In the controlled Class II cabinet environment, the solid warmer (LEC978) only fell by 0.5°C in the first 2 min before declining another 1.8°C after 5 more min but this was still more than the glass microscope stage warmer (MP30DM), which lost only 0.2°C over the first 2 min, and maintained a 1.0°C reduction for the next 5 min before a steady decline ensued.

Experiment B. Effect of Media and Oil Volume

Two stage warmers (MP30DM and LEC978) from Experiment A were used to compare the effect of the volume of culture media and the oil overlay on temperature maintenance (Fig. 2). The culture media volumes containing 1.0 mL of media with a small oil overlay are much more effective at maintaining temperature than 50 μL microdrops with 1.0 mL of oil overlay. This occurs with both stage warmers tested. The temperature loss occurs much more rapidly in both culture volumes in the LEC978 compared to the glass MP30DM.

Experiment C. Effect of Culture Vessel Lid and Media Volume

Figure 3 shows the use of culture dish lids and culture volumes on temperature change. The most effective stage warmer was chosen (MP30DM) and tested against both 50 μL droplet and 1.0 mL volumes with and without the presence of the culture dish lid. When a larger volume of media was used in conjunction with the dish lid, there was only a 0.3°C drop in 7 min. The 50 μL droplet with a lid lost 0.5°C within 3 min before steadily loosing temperature to 35.5°C after 7 min.

In dishes with the lid removed, the temperature loss was much greater at 3 min (1.5°C for 1.0 mL volume and 1.4°C for 50 μL volume), and the 1.0 mL volume continued to lose heat to 34.6°C after 7 min. The smaller volume 50 μL droplet appeared to begin absorbing heat from the stage warmer, as the heat loss stabilized around 35.4°C after 7 min.

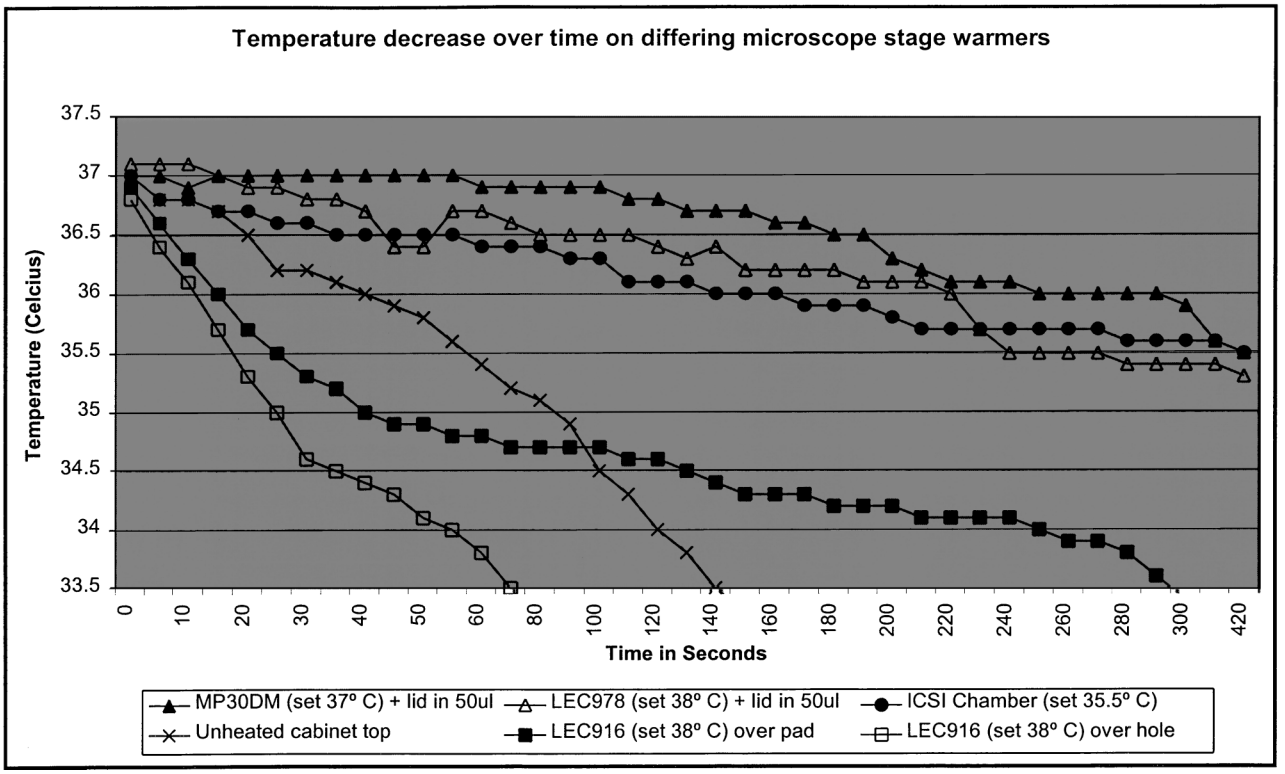

Fig. 1. Temperature decrease on differing microscope stage warmers, in 50 μL droplets.

Experiment D. Effect of Culture Incubator

The temperature regain from incubators using different heat dispersion principles (direct heat vs. air convection vs. absorbed heat) can be shown by Fig. 4.

The direct heat transfer incubator (MINC1000) regained the demand temperature of 37.0°C within 5.5 min for the 50 μL culture volume and within 6.5 min for the 1.0 mL culture volume. In comparison, neither culture volume placed into the large volume air convection incubator (FORMA) regained their demand temperature by 20 min (50 μL = 36.7°C; 1.0 mL = 36.2°C). Interestingly, both culture volumes that were placed into the air convection incubator actually lost further temperature before starting to regain. The 50 μL culture volume actually fell an additional 1.3°C before starting to warm up. The 1.0 mL culture volume fell only an additional 0.7°C; however, after 20 min had only reached 36.2°C compared to 36.7°C for the 50 μL culture volume. The temperature imparted by the Incuplate showed that it is a fairly useful intermediate method of transmitting heat to the surface of the culture vessel, and that it retains enough heat to not be overly affected by opening the large volume incubator door.

Since it absorbs heat from its surroundings, the maximum heat that was obtained from using the Incuplate for the 50 μL culture volume was only 36.7°C after 9.5 min, and for the 1.0 mL culture volume was 36.6°C, which it maintained after 17 min.

DISCUSSION

The results from these experiments demonstrate that temperature maintenance on an unprotected inverted microscope stage warmer can be very poor since the sample dish is exposed to ambient air currents from both air-conditioning and people about the laboratory. While these draughts are seemingly undetectable, they cause a culture dish on an inverted stage warmer to actually lose heat faster than a dish on an unheated bench-top in a controlled flow cabinet. Similarly, when viewing a culture droplet over a cut-away hole of the inverted microscope stage warmer, the temperature decrease was higher after 2 min compared to drops placed over the solid area of the same warmer. As expected the use of a heated perspex environmental chamber around the inverted microscope minimized the temperature loss. However, an

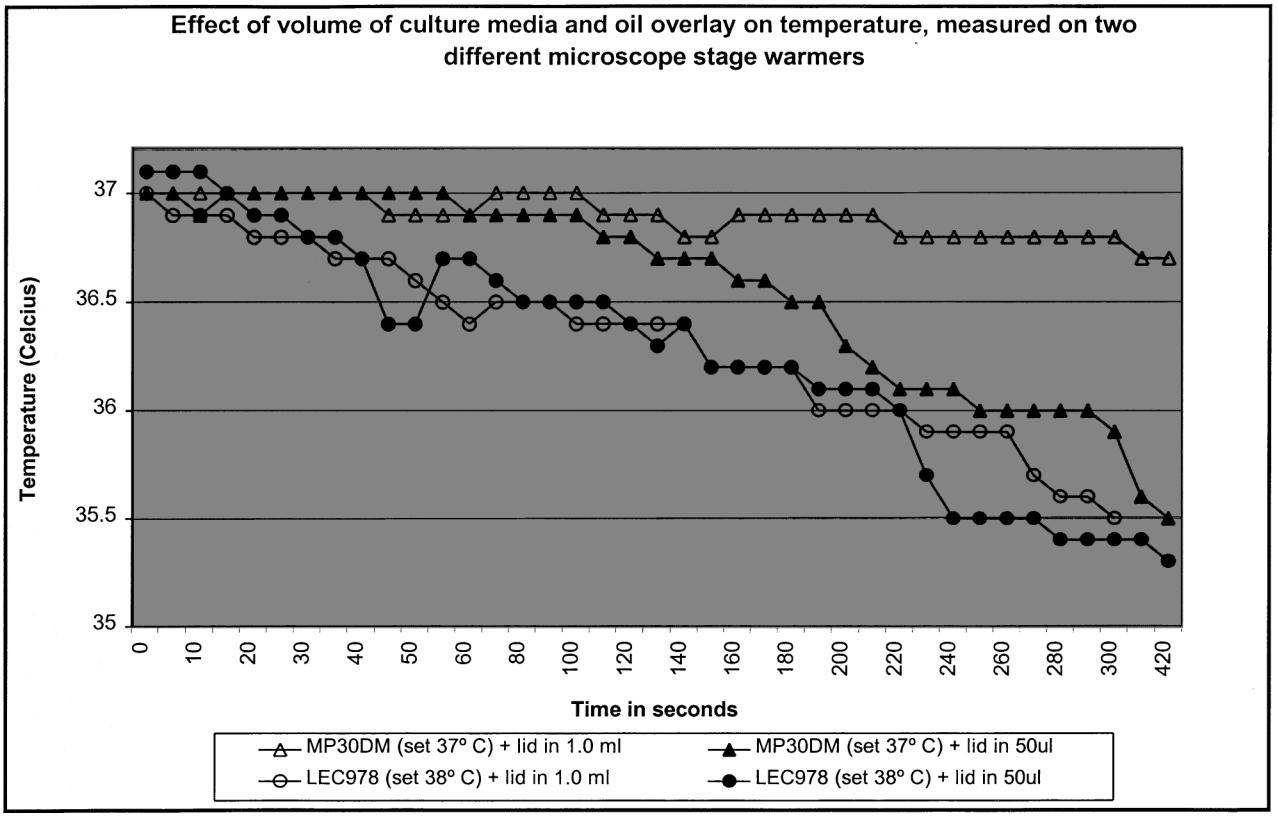

Fig. 2. Temperature drop with differing volume culture volumes (50 μL and 1.0 mL) and oil overlays (0.1 mL and 1.0 mL) on two microscope stage warmers.

environmental chamber is not perfect, as the blower for the heater is 12 cm from the thermocouple controlling the chamber temperature and 22 cm from the eventual position of the sample. While maintaining temperature at the set point of 35.5°C, the blower is releasing heat at 63°C!

The temperature decreases on microscope stage warmers are less pronounced when measured in a controlled air flow system (i.e. inside the Class II cabinet used for all oocyte/embryo manipulation). Here the influence of external circulating draughts is constant, but there is still a difference in temperature loss between different stage warmers. It is important to note that in all microscope warmers tested, the extent of temperature loss was only reduced but did not stop declining. Thus no stage warmer maintained the desired temperature or appeared to actually warm the culture media.

The results of the second experiment suggest that dogma proposing that large volumes of a viscous media such as oil act as an effective buffer to maintain temperature is unfounded, and the reverse is actually true. Larger volumes of media and smaller volumes of oil are more effective at maintaining temperature at the culture surface. This relationship held true for both stage warmers used. Perhaps the benefits of an oil overlay lie in antidesiccation and pH maintenance properties instead of temperature buffering capacity.

As might be expected, data from the third experiment showed there was a marked effect of buffering temperature loss when a culture vessel lid is left on. In fact, with the most effective stage warmer, the 50 μL droplet with the larger oil overlay and the 1.0 mL of media with the smaller oil overlay both maintained their heat much more effectively than the same volumes tested with their lids removed. Regardless of the stage warmer used or the presence or absence of culture vessel lids, the heat loss from a 50 μL droplet with an oil overlay is faster than that seen in 1.0 mL of media. After 3 min, the heat loss from the 1.0 mL of media without a culture vessel lid was not restricted by the use of the stage warmer, and the heat loss exceeded that seen for a 50 μL droplet without a culture vessel lid.

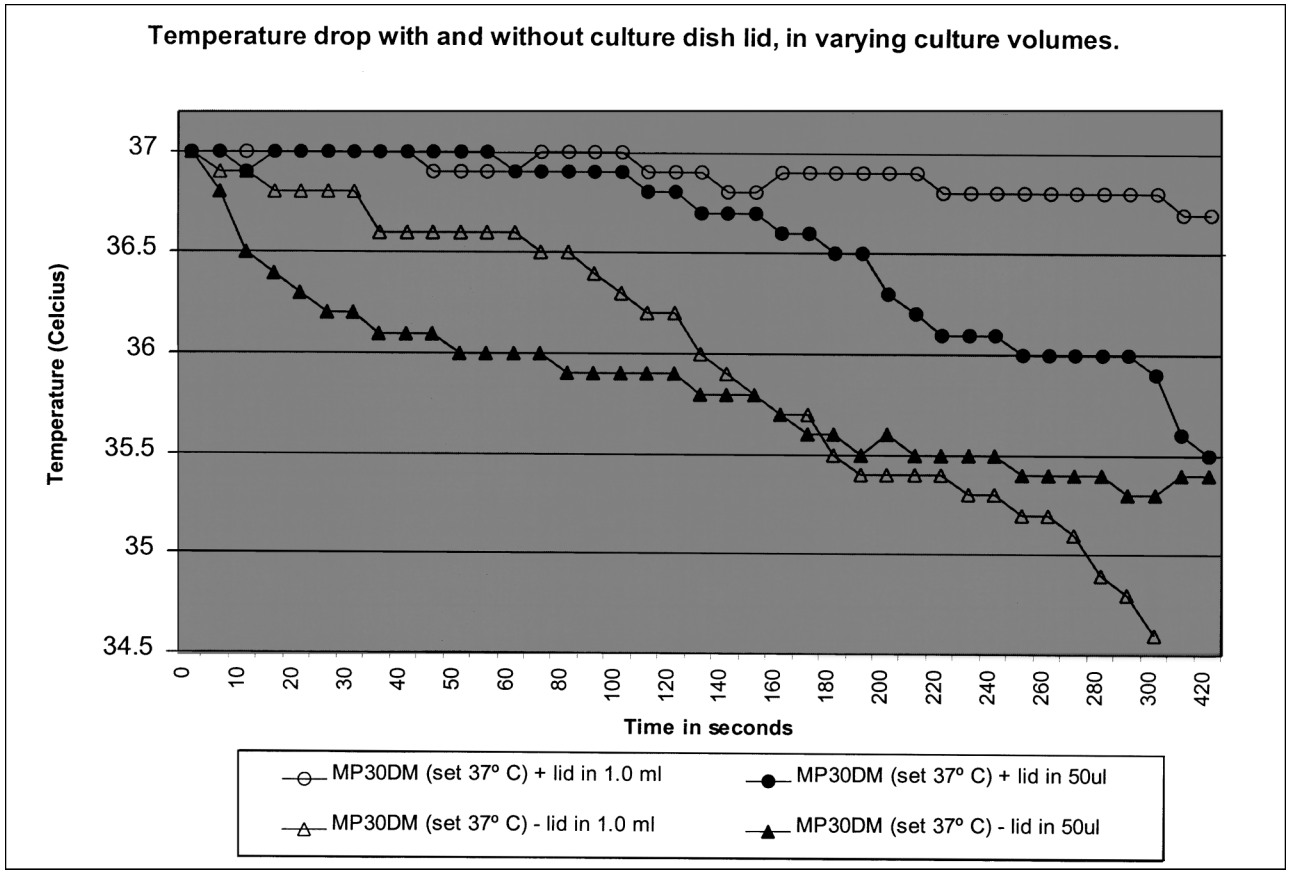

Fig. 3. Effect of culture dish lid and culture volume on temperature decrease using a single, microscope stage warmer.

This has implications for daily embryo checking in a laboratory. If the lids are left on and the embryo is removed for less than 2 min on an effective stage warmer, the temperature of the culture droplet does not change appreciably. If lids need to be removed, the 50 μL droplet falls to 36.0°C more than twice as fast as the 1.0 mL of media (50 s vs. 130 s).

The results from Experiment D demonstrated marked differences in the heating ability of different incubator systems. The direct heat incubator (MINC) was superior in warming culture media, and this was the only time in all the experiments where a return of temperature to set parameters was detected. The effect of the recesses in the MINC incubator surface allow the culture vessel surface to be in direct contact with the electrically controlled warm incubator surface, giving a very effective heat transfer mechanism. In comparison, the performance of the commonly used large volume air convection incubator (FORMA) was disappointing since it failed to return a culture vessel to set temperatures by 20 min. It was also worrying to note that this incubator only had its door opened once to place the dish inside during these experiments. In a routine human ART setting, it is probable that the incubator would be opened more than once in a 20-min period, indicating that the temperature measured in the culture media at the culture surface would not regain the set operating parameters for a considerable length of time. While the temperature decrease in the 1.0 mL volume in the large volume air convection incubator was buffered in comparison to that seen by the 50 μL droplet, if heat was lost, the larger volume took longer to reabsorb heat from the incubator chamber than the droplet did. Since the FORMA incubator has a large interior space to be heated, and the MINC has an extremely small space to be heated, the question arises as to whether the better temperature regulation of the MINC is due to contact with an active, electrically heated culture surface or due to the smaller air space to be heated. The Incuplate used is the same configuration as the culture surface of the MINC; however, the Incuplate

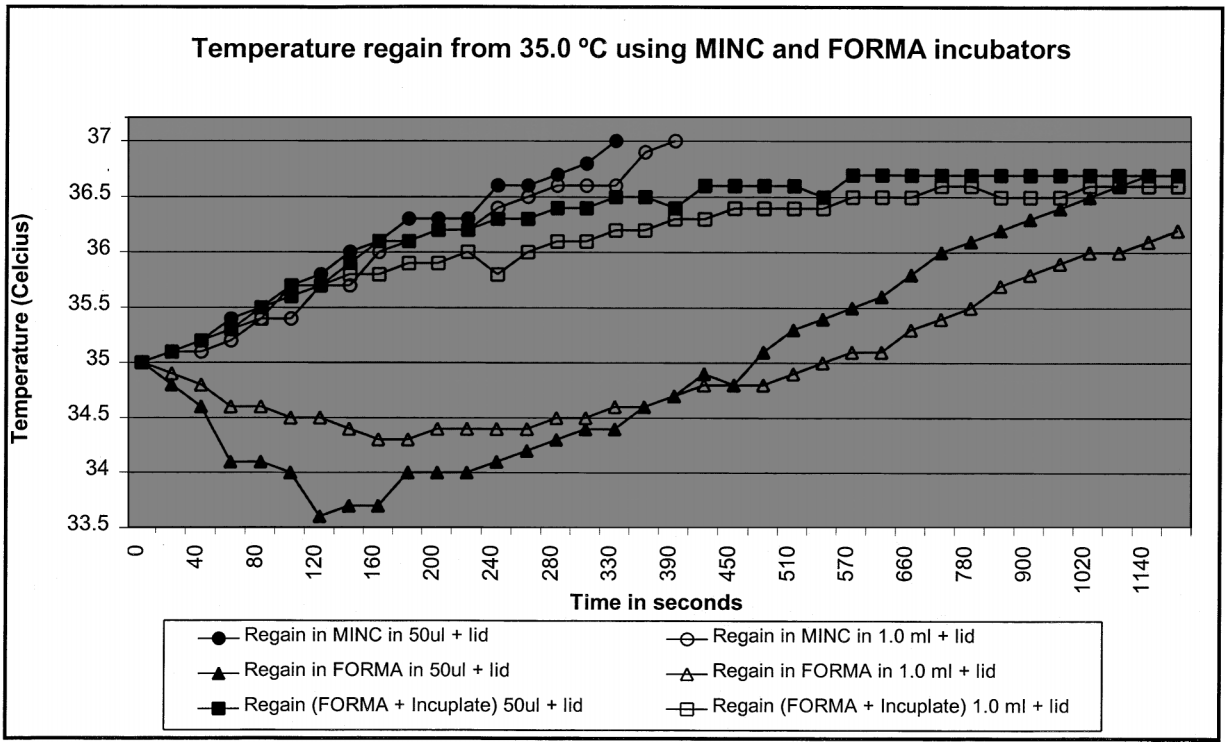

Fig. 4. Temperature regain between direct heat transfer incubator (MINC) and air convection incubator (FORMA), and an Incuplate thermal absorption insert with different culture volumes.

is not actively electronically heated, and it simply absorbs heat from its surroundings to donate to the culture vessel that is placed onto it. The temperature traces obtained showed that it is the active electrical control of the culture surface and not the decreased air space that makes the MINC so efficient at temperature regulation. The temperature loss and inability of either the FORMA or the Incuplate to return culture volumes to 37.0°C may provide evidence for the practice of changing embryos to new equilibrated culture dishes following several minutes of embryo viewing, instead of replacing these cooled dishes into an incubator and attempting to regain their heat.

Since there are some excellent pregnancy rates that are reported worldwide, it is possible that the heat retention devices or practices in successful clinics are better than in other clinics. It is also possible that some of the embryology practices that are mentioned in this paper may be currently in use (such as using differing media and oil volumes and the use of dish lids). By good design or good luck, warmers and incubators may also have been purchased that outperform other brands. Since the spindle of the oocyte may be affected to a greater degree by these uncontrolled temperature variations than the early embryo is, some of the embryology practiced in better performing clinics is probably serving to minimize any developmental damage to the oocyte and embryo.

The results of this practical study demonstrate that even though there is a perception in ART laboratories that temperature is controlled, there can be large, measurable differences between different temperature control systems. At no stage based on the type of warmer used, the presence or absence of a lid, or the volume of culture media or oil used, was the sample temperature actually maintained at 37.0°C for longer than 60 s, and only the magnitude of the heat loss was affected.

On the basis of these results, laboratory scientists can modify microscope equipment, culture volumes, viewing techniques, and most important the type of culture incubator used to minimize temperature loss during oocyte and embryo manipulation and culture.

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