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Comparison of Static and Dynamic Medium Environments for Culturing of Pre-implantation Mouse Embryos

Debra L. Hickman, DVM,[1] David J. Beebe, PhD,[2] Sandra L. Rodriguez-Zas, PhD,[1] and Matthew B. Wheeler, PhD[1,*]

The ability to manipulate mouse pre-implantation embryos in vitro has become a valuable tool in many scientific disciplines. However, fewer embryos maintain viability following in vitro manipulation compared with embryos in vivo. It has been suggested that use of dynamic medium environments may improve viability by simulating in utero environment. The objective of the study reported here was to compare a microdrop in vitro culture system with a microdevice in vitro culture system containing a static and two dynamic medium environments (0.1 and 0.5 μl/h) for culturing of mouse pre-implantation embryos. Results indicated that the static medium environment, in silicon-glass microdevices, was not significantly different from the microdrop control environment in proportion of embryos developing from the two-cell to the blastocyst stage. However, the static microdevice environment produced significantly ($P < 0.05$) more morulas than did that of the control group. Both of these treatment groups, under the presented conditions, consistently had significantly higher proportions of blastocysts ($P < 0.05$) and morulas ($P < 0.05$) and lower proportions of abnormal ($P < 0.05$) and eight-cell embryos ($P < 0.05$), compared with those of the high flow rate dynamic environment microdevice treatment groups. Studies exploring slower or pulsatile rates of medium delivery in a dynamic medium environment are indicated.

In vitro manipulation of mouse pre-implantation embryos has become a valuable research tool for reproductive physiologists and embryologists. It also is an integral part of the development of transgenic animal models. However, the production of appropriate quantities of viable embryos for transfer after manipulation has been an elusive challenge. Depending on the strain of mouse and in vitro manipulation procedure performed, the percentage of early cleavage embryos that develop to morula and blastocyst can range from 20 to 40% (1, 2). It has been hypothesized that numerous variables associated with the in vitro system (including accumulation of metabolites, such as lactate and ammonia and depletions of necessary nutrients) are the cause of the poor yields of viable embryos in vitro (3). In the in vivo environment, the female reproductive tract is constantly active, resulting in movement of the embryo down the reproductive tract (4). This movement also washes the embryos in changing fluid in the uterine tube and uterus, possibly maintaining a gradient for the supply of necessary nutrients and the removal of waste products (4). As this in vivo environment is dynamic, it suggests that use of a perfusion system may be beneficial in overcoming limitations of static culture (3).

Dynamic in vitro culture of cells has been used extensively in multiple fields of biology (e.g., 5-7). However, it has been largely overlooked in embryo culture because it has proven challenging to create a system that allows the embryos to be moved minimally without damage to them while maintaining the appropriate pH and carbon dioxide concentration (8-12). Tissue culture systems that pump the medium through the embryo chamber or through a chamber adjacent to the embryos have been used, with minimal success (8-12). Turbulence and flow rates > 50 μl/h, causing depletion of nutrients and the removal of required hormones and growth factors, have been proposed as factors contributing to this developmental failure (8, 9). Ideally, dynamic culture would evolve to mimic the conditions of the oviduct and uterus, yielding a higher number of viable blastocysts, comparable to the in vivo situation. Dynamic culture also would allow collection and analysis of culture fluids for detection of markers of embryo viability.

The development of miniaturization technologies toward small mechanical systems, microelectromechanical systems (MEMS), has created a means for dynamic culture on a volumetric scale reportedly more consistent with the needs of the embryo (3, 13). To address concerns cited in the previously used cell culture systems we previously developed a silicon-glass microdevice for in vitro manipulation of pre-implantation embryos, using MEMS technology (14). The first objective of the study reported here was to determine whether use of an in vitro embryo manipulation microdevice system (14) would result in a higher percentage of viable blastocysts, compared with results using the more common microdrop in vitro culture method. The second objective of the study was to explore the effect of dynamic flow on development of mouse pre-implantation embryos in silicon-glass microdevices.

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[1]Department of Animal Sciences, University of Illinois, 1207 West Gregory Drive, Room 366, Urbana, Illinois 61801, and [2]Department of Biomedical Engineering, University of Wisconsin, Madison, Wisconsin, 53706.
[*]Corresponding author.

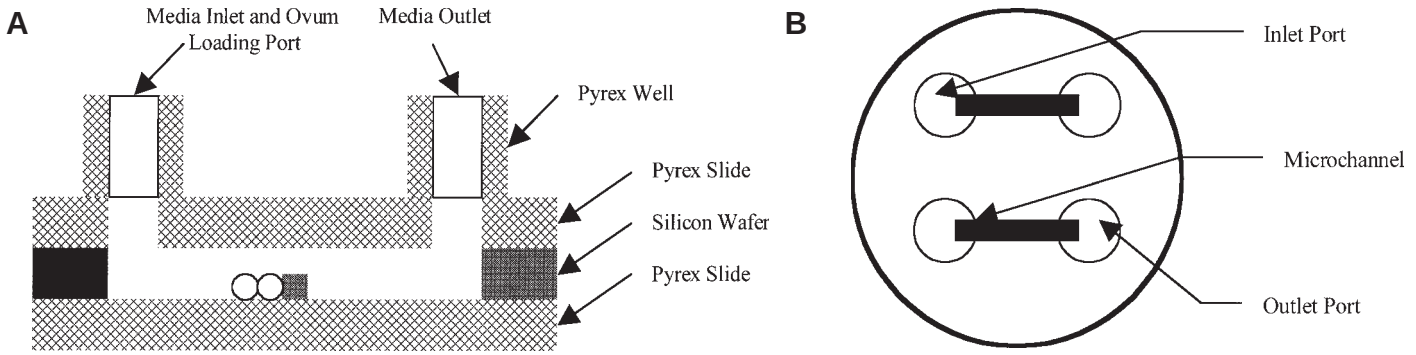

Figure 1. Schematic cross-section (A) and top-down view (B) of the silicon-Pyrex microdevice. The basic structure of the device is a sandwich with Pyrex 7740 slides forming the bottom and top layers.

Materials and Methods

Mice. Female ICR mice, at least 3 weeks old, were obtained from Jackson Laboratory (Bar Harbor, Maine) for use as donor females. The SB6F1 male mice, obtained by breeding SJL/J females from Jackson Laboratory with C57BL/6J males from Jackson Laboratory were used as stud males to produce embryos. All animals were housed under environmental conditions of 22°C, 40 to 70% humidity, 15 air changes/h, and a 14:10-hr light:dark cycle. Animals were housed in polycarbonate microisolation cages (7.5 × 11.5 × 5 in.) with filter tops. The bedding was composed of autoclaved heat-treated hardwood chips (Sanichips, P. J. Murphy Inc., Montville, N.J.). Mice were fed autoclaved LM-485 (Diet 7012) Mouse/Rat Sterilizable Diet (Harlan Teklad, Madison, Wis.) and autoclaved water ad libitum. Sentinel animals were tested quarterly, and found to be test negative, for mouse hepatitis virus, Sendai virus, pneumonia virus of mice, mouse parvovirus, minute virus of mice, mouse encephalomyelitis virus, reovirus type 3, lymphocytic choriomeningitis virus, ectromelia, mouse rotavirus, *Mycoplasma pulmonis*, and rodent pinworms. The UIUC Animal Care and Use Committee approved all animal experiments.

Preparation of microdrop in vitro culture system. Embryos were cultured in a 60 × 15-mm tissue culture dish (Becton Dickinson Labware, Franklin Lakes, N.J.) in a 35-µl drop of M16 culture medium (15) under 5 ml of embryo-tested mineral oil (Sigma Chemical Co., St. Louis, Mo.). The mineral oil was washed with double-deionized water (Barnstead, Dubuque, Iowa) and filtered through a 0.22-µm syringe filter unit (Millipore Corp., Bedford, Mass.) prior to use in the laboratory to further ensure removal of potential contaminates. The system was equilibrated at 37°C in a 5% CO_2/air atmosphere prior to use.

Manufacture of the microdevices. The fabrication process has been described elsewhere (14). In brief, the microdevice consists of a processed silicon wafer between two pieces of Pyrex 7740 glass, with Pyrex wells adhered. The inlet well is wide to facilitate placement of the embryos. The outlet well is luer-lock adapted to allow medium to be drawn out. These devices have two to three parallel transparent microdevices (250 µm deep and 1 mm wide) etched in the wafer and are designed for group culture of pre-implantation embryos (Fig. 1).

Preparation of static environment microdevices. Microdevices were cleaned ultrasonically, rinsed with 70% ethanol and double-deionized water (Barnstead), and autoclaved between replicates. The autoclaved microdevices were loaded by placing 0.5 ml of M16 culture medium (15) in the inlet well. A tuberculin syringe (Becton Dickinson & Co., Franklin Lakes, N.J.) was attached to the luer-lock adapter over the outlet well. Using the syringe, gentle pressure was applied to pull medium from the inlet to the outlet well. The total volume of M16 culture medium (15) in the devices was 0.5 ml, including that in the inlet and outlet wells. Mineral oil was not used with these devices. The system was equilibrated at 37°C in a 5% CO_2/air atmosphere prior to use.

Preparation of dynamic environment microdevices. The microdevices were prepared as described for the static environment. All plastic equipment was cleaned with 70% ethanol and double-deionized water (Barnstead) and was gas sterilized with ethylene oxide between replicates. Hamilton syringes (Reno, Nev.) were cleaned with 70% ethanol and double-deionized water (Barnstead) and were autoclaved between replicates. The M16 culture medium (15) used in the dynamic culture system was equilibrated at 37°C in a 5% CO_2/air atmosphere prior to use. A three-way stopcock (Sigma Chemical Co.) was placed on the female end of the polyvinyl chloride tubing (Cole-Parmer Instrument Co., Vernon Hills, Ill.). A two-way stopcock (Cole-Parmer Instrument Co.) was placed on the male end of the tubing. A blunted 18-gauge needle (Becton Dickinson & Co.) was placed on the male end of the two-way stopcock. A 20-ml syringe (Becton Dickinson & Co.) and 0.22-µm syringe filter unit (Millipore Corp.) were used to load approximately 5 ml of M16 culture medium (15) into the tubing, taking care to remove all air bubbles from the tubing. For each channel, two lengths of tubing were prepared. One had a 50-µl Hamilton syringe filled with M16 culture medium (15) connected to the three-way stopcock. The second had an empty 50-µl Hamilton syringe attached to the three-way stopcock.

The Hamilton syringes were placed in a PHD 2000 Infusion/Withdraw syringe pump (Harvard Apparatus, Holliston, Mass.). Entering the diameter of the Hamilton syringes and the desired rate of flow into the control panel programmed the syringe pump. This pump has the capability to produce flow between 0.0001 µl/h and 220.82 ml/min.

The ends of the tubing with the blunted 18-gauge needles were introduced into the incubator (Forma Scientific Inc., Marietta, Ohio) via a rear access port. The tubing was run through holes drilled in a rubber stopper that was placed in the port to help seal the incubator chamber. After loading the embryos into the microdevices, the blunted 18-gauge needle con-

nected to the tubing with the full Hamilton syringe was placed in the input well of the microdevice. The blunted 18-gauge needle connected to the tubing with the empty Hamilton syringe was placed in the output well of the microdevice. The infusion protocol was initiated by commanding the syringe pump to run the entered program.

Preparation of embryos. Three- to eight-week-old ICR female mice (Jackson Laboratory) were superovulated by intraperitoneal (i.p.) administration of an injection of 15 IU of pregnant mare serum globulin (PMSG; Sigma Chemical Co.) 92 to 96 h prior to collection, followed by i.p. administration of 10 IU of human chorionic gonadotropin (hCG; Sigma Chemical Co.) 44 to 48 h prior to collection. The females were placed with SB6F1 studs immediately following the hCG injection. The presence of a vaginal plug the following morning (day one of pregnancy) indicated that mating had taken place at the midpoint of the dark cycle. Forty-four to 48 h after hCG injection, the ICR female mice were euthanized by CO_2 asphyxiation. The oviducts were dissected out and placed into M2 collection medium (15). Two-cell embryos were flushed out of the oviducts with M2 medium (15). They were washed once in the M2 medium (15) and three times in the M16 culture medium (15), prior to distribution into treatment groups.

Experimental design. Embryos cultured in vitro in the microdrop were considered the control group for this experiment. Previously reported dynamic flow systems had used flow rates > 50 µl/h and conditions of turbulence without success (8, 9). To begin to target the ideal rate of flow, we decided to attempt a 10×, 100×, and 500× decrease in previously reported flow rates for the microdevices. The design of the microdevices creates conditions of laminar flow, without turbulence (14). Three treatment groups were established: in vitro culture in the microdevice under static medium conditions, in vitro culture in the microdevice under dynamic medium conditions at 0.1 µl/h, and in vitro culture in the microdevice under dynamic medium conditions at 0.5 µl/h. After collection, groups of 15 two-cell embryos were placed in microdrop treatment and microdevice treatment groups, respectively. The embryos were allotted to treatment groups, using a randomized block design. An equal number of embryos from each donor were placed in each treatment group to balance the heterogeneity of embryos and distribute embryos from each donor. All embryos were maintained in an incubator (Forma Scientific Inc., Marietta, Ohio) for 72 h. The dishes and microdevices were removed from the incubator at 24-h intervals to record the stages of development by examination under a dissecting microscope. The embryos were classified as four-cell, eight-cell, sixteen-cell embryo, morula, blastocyst, or abnormal (degenerating) embryo. The same operator performed evaluation of the stages of development at each 24-h interval. The dishes and microdevices were taken out and put back in the incubator at the same time to eliminate variation among groups. This was done as quickly as possible to eliminate excess variation in culture conditions. Replications were done over days and donors to segregate donor variability from the treatment effect.

Results

Embryo culture. The stages of development (abnormal, eight-cell, and 16-cell embryos, morulas, and blastocysts) at each observation (24, 48, and 72 h of culture) were analyzed, using the PROC PROBIT procedure in SAS (SAS Institute Inc., Cary, N.C.). The model included the fixed effects of replicate, treatment, and time, and the time-treatment interaction. For each analysis, when a factor was not significant, ($P > 0.05$), it was subsequently removed from the model. In all instances, replicate was found to be non-significant and was removed from the model.

The initial PROC PROBIT analysis examined the proportion of each type of embryo observed in each treatment group. Eight-cell embryos, morulas, blastocysts, and abnormal embryos were analyzed with respect to treatment (Fig. 2).

After 72 h of culture, the static environment microdevice treatment and control groups were not significantly ($P > 0.05$) different with regard to the proportion of embryos, except at the morula stage of development. The static environment produced significantly ($P < 0.05$) more morulas than did that of the control group. The static environment microdevice treatment group consistently had significantly higher proportions of blastocysts ($P < 0.05$) and morulas ($P < 0.05$) and lower proportions of abnormal ($P < 0.05$) and eight-cell embryos ($P < 0.05$), compared with the dynamic environment microdevice treatment groups. The two dynamic environment microdevice treatment groups had no significant differences in the proportion of embryos at any stage of development when compared with each other ($P > 0.05$).

Time and treatment interactions were also analyzed within each developmental stage of embryo. Significant time and treatment interactions were not found for blastocysts or abnormal cells. Significant time and treatment interactions were found for morulas and eight-cell embryos (Fig. 3).

At 24 h, the 0.1- and 0.5-µl/h dynamic environment microdevice treatment groups had significantly fewer eight-cell embryos ($P < 0.05$), compared with the control and static environment microdevice treatment groups. The static environment microdevice had a significant decrease in the proportion of morulas seen at 24 h ($P < 0.05$), compared with the control group. The 0.1-µl/h dynamic environment microdevice treatment group also had a statistically significant decrease in the proportion of morulas seen at 24 ($P < 0.05$) and 48 ($P < 0.05$) h, compared with the control treatment group. Other significant interactions were not observed.

An additional treatment group, 5.0 µl/h, was studied, but was not included in the statistical analysis. Only two successful replicates of this treatment group were performed. The blastocyst stage of development was not achieved in either of these replicates and after 72 h of culture, and the proportion of abnormal embryos was 100%. It was concluded that this rate of flow for 72 h was detrimental to development of mouse pre-implantation embryos. However, examination of the raw data revealed a higher percentage of morulas at the 24-h stage of in vitro culture, compared with that of the other treatment groups (Fig. 4).

Discussion

Since the practical use of in vitro manipulation of embryos is to produce usable offspring, the gold standard for successful in vitro development is the production of pups after embryo transfer. The desired developmental stages after in vitro embryo culture are morulas and blastocysts, as these are the developmental stages suggested for successful mouse embryo transfer (15). Analysis of the in vitro culture data does not indicate advantage to the static environment silicon-glass microdevice, compared with the microdrop culture, for the production of blastocysts since both of these treatment groups produced similar proportions of blastocysts. There may be an advantage to the static environment microdevice treatment group in development to the

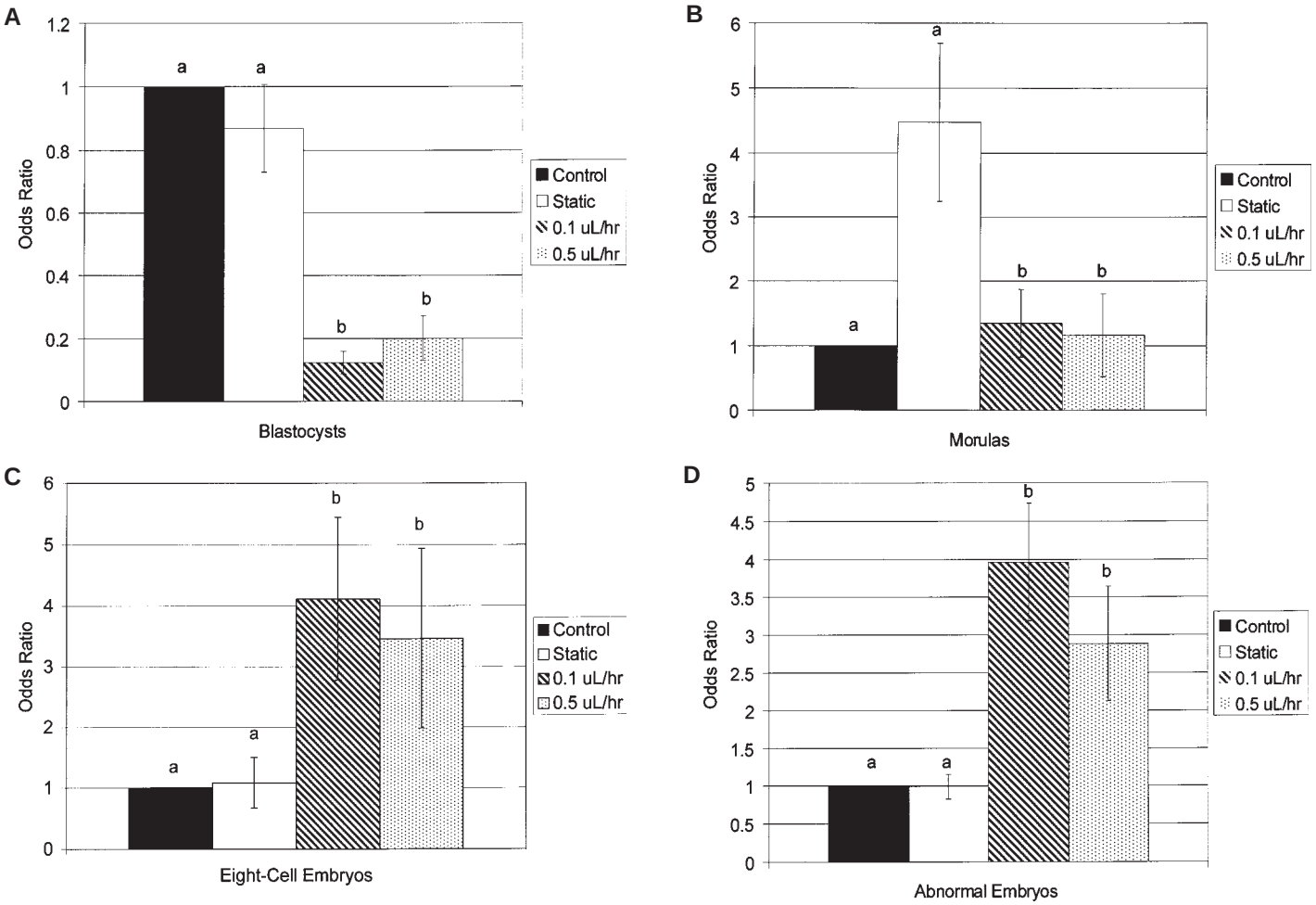

Figure 2. Odds ratios for (A) blastocysts, (B) morulas, (C) eight-cell embryos, (D) and abnormal embryos, relative to control values by individual treatments. Error bars indicate standard error for odds ratio. Different letters denote significant differences ($P < 0.05$).

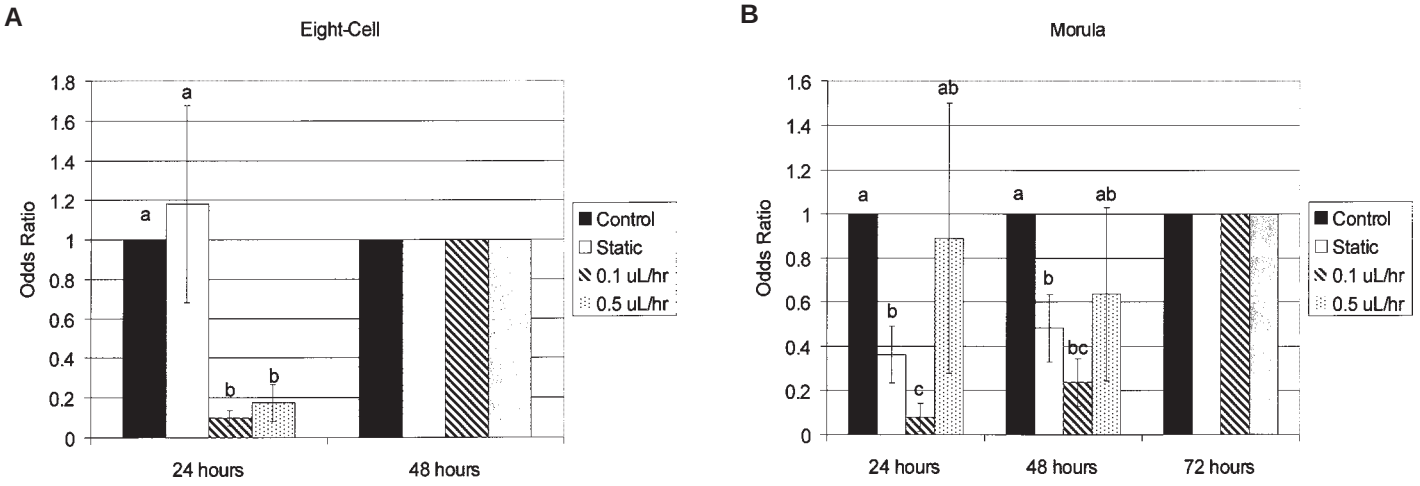

Figure 3. Time-treatment interactions for (A) eight cell embryos and (B) morulas. Different letters denote significant differences ($P < 0.05$).

morula stage.

It also is clear that the specific dynamic medium environments selected for these experiments are not conducive to development of mouse pre-implantation embryos. This conclusion is supported by the observation that use of both selected dynamic environment microdevice treatment groups resulted in significantly lower proportions of embryos of the blastocyst and morula developmental stages. However, this does not mean that all dynamic flow rates in microdevices are contraindicated and inhibit embryo development. On the contrary, the present re-

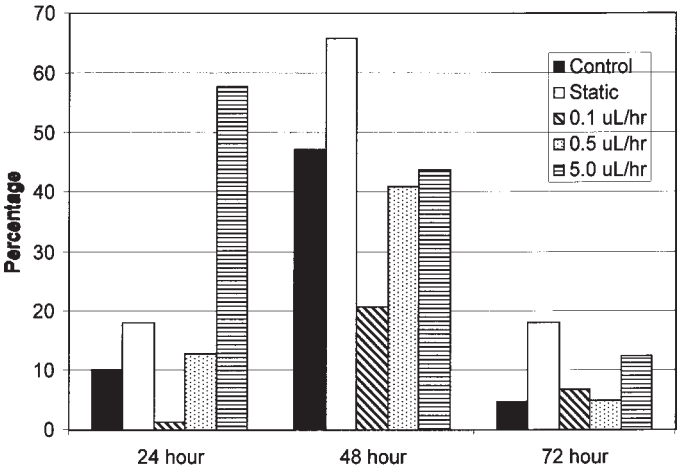

Figure 4. Raw data percentage of morulas at given times for each treatment group.

sults suggest that investigation into additional and possibly slower flow rates is definitely warranted and is currently being investigated in the laboratory.

Although an examination of time and treatment interactions revealed significant interactions at the eight-cell and morula stages of development, there were not consistent advantages in one treatment over another. The proportion of eight-cell embryos in the static environment microdevice treatment group was not significantly different from the proportion for the control group, as indicated by the time and treatment interactions. The proportion of morulas for the static environment microdevice treatment group was significantly higher than that for the control group. The development in the specific dynamic medium conditions described was not better, and often was worse, than that seen for the static environment microdevice and the control treatment groups. A potential exception to this was the observation in the raw data that the embryos of the 5.0 µl/h dynamic environment microdevice treatment group had an extremely high proportion of morulas at the 24-h time point, compared with that for the other treatment groups. It has been suggested that the metabolic requirements of the early pre-implantation embryo are increased (e.g., 3, 9-12), suggesting that an initial rapid supply of nutrients and removal of waste products could facilitate development. This potential early beneficial effect requires further exploration.

There are multiple reasons why the dynamic environment microdevice groups may have behaved as poorly as they did, compared with the control and static environment microdevice groups. It is possible that these rates of flow were still too high for mouse pre-implantation embryos, washing away necessary compounds in addition to waste products. This effect could be corrected through use of slower or pulsatile flow rates. It is also possible that the incubator had a negative effect on culturing of the embryos (16). Although it was assumed that the pH of the medium introduced into the microdevice was constant (diffusion through the polyvinyl chloride tubing maintained in the incubator), it is possible that this was not the case. It may be valuable to analyze the pH of medium within the tubing. One factor that has reportedly caused problems for dynamic in vitro culture of pre-implantation embryos that can be confidently dismissed is turbulence caused by the medium flow since the microdevice system produces laminar flow (14).

In conclusion, the static environment in the silicon-glass microdevice system was significantly ($P < 0.05$) different from the control environment for the production of morulas, but was not significantly different for the production of blastocysts from two-cell embryos. Both of these treatment groups performed significantly better in the percentage of embryos that developed into blastocysts than did the selected dynamic medium environment groups. However, the suggestive benefit of a rapid dynamic medium environment in the first 24 h, illustrated by the 5.0-µl/h treatment group, warrants additional study. Further studies exploring slower or pulsatile rates of media delivery in a dynamic medium environment are indicated.

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