Reproductive BioMedicine Online (2012) **24**, 142–147

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ARTICLE

Microdrop preparation factors influence culture-media osmolality, which can impair mouse embryo preimplantation development

JE Swain [a,d,*], L Cabrera [a], X Xu [a], GD Smith [a,b,c,d]

[a] *Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI 48108, United States;* [b] *Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI 48108, United States;* [c] *Department of Urology, University of Michigan, Ann Arbor, MI 48108, United States;* [d] *Reproductive Sciences Program, University of Michigan, Ann Arbor, MI 48108, United States*

[*] Corresponding author. *E-mail address:* swainj@med.umich.edu (JE Swain).

Jason Swain, PhD, HCLD is an assistant professor in obstetrics and gynaecology, member of the reproductive sciences programme and scientific director of Assisted Reproduction Technology Laboratories at the University of Michigan. He completed his BSc at Hillsdale College in his native Michigan, his MSc in animal science at Purdue University with Dr Rebecca Krisher, his PhD in molecular and integrative physiology at the University of Michigan with Dr Gary Smith and his post-doctoral training with Dr Thomas Pool. Jason's research interests include mechanisms involved in oocyte maturation, focusing on the role of reversible phosphorylation and of improving in-vitro embryo culture conditions.

Abstract Because media osmolality can impact embryo development, the effect of conditions during microdrop preparation on osmolality was examined. Various sizes of microdrops were prepared under different laboratory conditions. Drops were pipetted directly onto a dish and covered by oil (standard method) or pipetted on the dish, overlaid with oil before removing the underlying media and replaced with fresh media (wash-drop method). Drops were made at 23°C or on a heated stage (37°C) and with or without airflow. Osmolality was assessed at 5 min and 24 h. The biological impact of osmolality change was demonstrated by culturing 1-cell mouse embryos in media with varying osmolality. Reduced drop volume, increased temperature and standard method were associated with a significant increase in osmolality at both 5 min and 24 h (*P*-values <0.001, <0.0001 and <0.0001, respectively). There was a significant interaction between airflow, decreased volume, increased temperature and standard method that caused a significant increase in osmolality (40 mOsm/kg) compared with controls ($P < 0.04$). There was no significant change in osmolality over time. Mouse embryo development was significantly reduced in media with elevated osmolality (>310 mOsm/kg; $P < 0.05$). Procedures in the IVF laboratory can alter osmolality and impact embryo development.

KEYWORDS: blastocyst, embryo, environmental stress, microdrop, osmolality

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2011.10.008

Introduction

Commercial production of embryo culture media has led to greatly reduced variability in media formulations and contributed to the overall improvement in embryo development *in vitro*. However, while there is improved consistency in media ingredients and quality, there are still aspects of culture media that are controlled directly within individual laboratories that dramatically influence resulting embryo development. These laboratory-controlled variables underscore the need for strict attention to detail in laboratory procedures and protocols. For example, altitude of the laboratory, temperature, incubator atmosphere, amount and type of protein supplement and other factors can all influence media pH, which may result in the same media having different pH in different laboratories. Perhaps more importantly, certain media characteristics, although initially set by the commercial manufacturer, can still be influenced within individual laboratories through common practices, sometimes unintentionally and unknowingly.

One media characteristic that is set by commercial media manufacturers is osmolality. Osmolality is a measure of solute particles dissolved in a solution as calculated by an osmometer. This value, regardless of whether the osmometer uses vapour pressure or freezing-point depression technology, is reported in mOsm/kg. Osmolality, although similar, is not the same as the less accurate osmolarity, which is calculated in terms of solvent volume and is thus dependent upon temperature and reported as mOsm/l (Erstad, 2003). Regardless, both osmolality and osmolarity are important because they represent a measure of solute concentration, which can exert osmotic pressure, subsequently impacting cell volume and thus acting as a stressor and impacting embryo growth/function. Importantly, mammalian preimplantation embryos can develop over the osmolality range of ~255–295 mOsm/kg, while osmolalities outside this range impair embryo development, although this can be media dependent (Brinster, 1965; Dawson and Baltz, 1997; Hay-Schmidt, 1993; Liu and Foote, 1996; Miyoshi et al., 1994; Richards et al., 2010).

Initially set by commercial media manufacturers, osmolality is an especially important media characteristic because it can inadvertently be altered in the laboratory. Evaporation, or factors facilitating evaporation, can alter media osmolality and embryo development. Thus, conditions used during media preparation or techniques utilized during the process could have an impact. Factors such as volume of media, temperature of preparation surface and time used in preparation, could lead to osmolality shifts. Therefore, the objective of this study was to examine a variety of conditions used during media preparation and quantify the impact on resulting media osmolality, as well as determine the impact of resulting osmolality shifts on mouse preimplantation embryo development.

Materials and methods

Microdrop preparation

Microdrops of P1 medium (Irvine Scientific, Santa Ana, CA, USA) + 10% SSS or Serum Substitute Supplement (Irvine Scientific) were prepared on 60 mm polystyrene dishes under a variety of conditions to determine the impact of: (i) media volume (10, 20 and 40 μl); (ii) airflow (on and off); (iii) work-surface temperature (37°C and ~23°C); and (iv) preparation method (standard or wash drop) on resulting osmolality (24 experimental groups; **Figure 1**). Standard microdrop preparation involved pipetting the media on the dish and covering with oil, while the wash-drop method started as the standard method, but then medium was removed from under the oil overlay using a small pipette and then replenished with fresh medium.

Fifteen drops were dispensed in each dish, with a pair of dishes for each combination of media volume, airflow and temperature conditions made at a time. This resulted in ~1.5 min elapsing before being covered with 7 ml unwashed mineral oil (Irvine Scientific). Each of the 24 experimental groups were sampled at both 5 min and 24 h, resulting in a total of 48 sets of conditions (**Figure 2**). Dishes for each experimental group were made in triplicate.

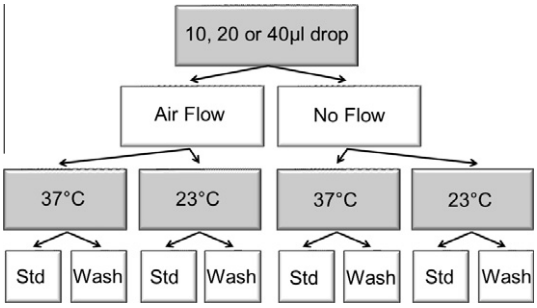

Figure 1 Schematic illustrating experimental design and resulting groups. Microdrops of three different volumes were tested. Drops were made in a sterile hood with or without airflow, with or without a heated surface and using one of two preparation methods. This resulted in eight groups tested for each microdrop volume (24 experimental groups). Media were sampled at both 5 min and 24 h, resulting in a total of 48 endpoint measurements. Each measure was performed three times over three replicates and the mean of the nine measurements taken. Std = standard method; Wash = wash-drop method.

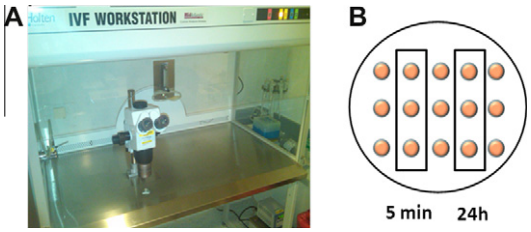

Figure 2 (**A**) Dishes were made in an IVF workstation with laminar airflow turned on or off and with the heated working surface at 37°C or ~23°C. (**B**) Example of microdrops on a dish. Fifteen microdrops were aliquoted per dish (with two dishes made up before adding oil overlay). Microdrops were sampled at 5 min and 24 h after preparation.

Osmolality measurement

Approximately 10 μl from each microdrop prepared under each condition was carefully removed and loaded onto a precalibrated osmometer (Vapro5520 Osmometer; Wescor, Princeton, NJ, USA). Three microdrops were measured from each dish, which were made in triplicate. Osmolality values were averaged from nine measurements (three per dish × three triplicate dishes).

Embryo development

To demonstrate the impact of osmolality on embryo development, media were formulated with osmolalities that covered the ranges obtained in prior experiments. Varying osmolality (270, 290, 310 and 330 mOsm/kg) were formulated by adjusting (decreasing/increasing) the NaCl concentration of human tubal fluid media (HTF; Quinn et al., 1985). HTF was selected because of the absence of amino acids. Media was supplemented with 3% human serum albumin (Irvine Scientific). Ten frozen–thawed 1-cell mouse embryos (F1 hybrid; B6C3F-1 × B6D2F-1; Embryotech, Haverhill, MA) were cultured (ten per 50 μl drop) and covered with 7 ml mineral oil. Embryos were cultured at 37°C in ~6% CO_2 in air (pH 7.27–7.32) and development was assessed at various time points over 96 h. Data were collected from three replicates.

Statistical analysis

Statistical analyses were performed using SAS statistical software (Cary, NC, USA) or SPSS (Chicago, IL, USA). Osmolality measurement outcomes were analysed using bivariate analysis looking at impact of volume, airflow, temperature and drop preparation technique on media osmolality. Data are expressed as osmolality increase over controls (media out of the bottle). Multivariate analysis was used to examine interactions between environmental conditions and impact on osmolality. For appropriate comparison, controls consisted of the experimental condition that yielded the lowest osmolality change (40 μl drops, no airflow, 23°C, wash-drop technique). Differences were analysed using *t*-test or ANOVA. Absolute osmolality measures between treatments were analysed using ANOVA and Bonferonni. Embryo developmental data were analysed using ANOVA and Bonferonni multiple comparison test. All data are expressed as mean ± SEM.

Results

Preparation of microdrops under different conditions affected media osmolality. Bivariate analysis of media sampled 5 min after microdrop preparation over all experimental conditions demonstrated that drop volume, temperature and method all significantly affected osmolality (**Figure 3**). Preparation at 37°C significantly increased osmolality compared with preparation at 23°C ($P < 0.0001$). Preparation of 10 μl drops significantly increased osmolality compared with 20 or 40 μl ($P < 0.001$). Standard drop preparation significantly increased osmolality compared with the wash-drop method ($P < 0.0001$). No differences in media osmolality were observed with airflow alone. The same differences were apparent following 24 h of culture and there were no significant differences in osmolality between 5 min and 24 h, indicating that unwashed oil did not facilitate osmolality increases (data not shown).

Subsequent multivariate analysis compared osmolality of microdrops prepared in various experimental conditions to those obtained from 40 μl microdrops prepared using no airflow, at 23°C and the wash-drop method, selected as the control group as these conditions yielded the lowest osmolality change compared with that of media out of the bottle. Statistical analysis confirmed prior bivariate analysis that drop volume, temperature and drop preparation method significantly impact media osmolality. The only statistically significant interaction observed was when all parameters were changed to 10 μl drop volume, airflow, 37°C and standard method, resulting in a significant increase in media osmolality ($P < 0.0001$).

To further quantify media osmolality changes between experimental conditions and reinforce the amount of shift that can occur under normal laboratory conditions, osmolal-

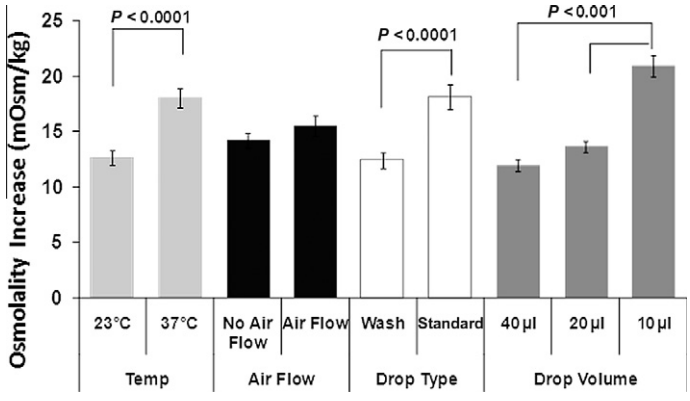

Figure 3 Bivariate analysis over all experimental groups demonstrates that conditions used during microdrop preparation impact media osmolality. Data presented are representative of sampling after 5 min. Lower drop volume, higher temperature and the standard method all significantly increased media osmolality. No differences were apparent after 24 h. Data are mean ± SEM increase over control media sampled out of the bottle.

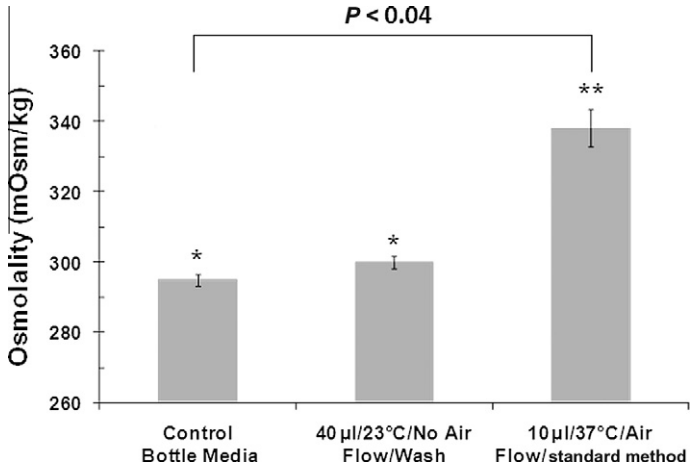

Figure 4 Comparison of conditions used during microdrop preparation that resulted in the lowest and largest change in osmolality compared with control media out of the bottle. Smaller volume, airflow, increased temperature and the standard method resulted in a ~40 mOsm/kg increase in osmolality. Different superscripts between treatments represents a statistically significant difference.

ity of media sampled out of the bottle was compared with that of microdrops prepared under conditions that resulted in the lowest and greatest amount of osmolality shift, as indicated by the multivariate analysis (lowest shift: 40 µl, no airflow, 23°C, wash-drop method; highest shift: 10 µl, airflow, 37°C, standard method). Microdrops of 10 µl prepared with airflow at 37°C and the standard method resulted in a significant increase in osmolality (~40 mOsm/kg) compared with media out of the bottle or 40 µl drops prepared with no airflow at 23°C and the wash-drop method ($P < 0.04$; Figure 4).

To determine the biological impact of media osmolality changes obtained in the laboratory under different experimental conditions, development of mouse embryos was examined in media that covered the osmolality range obtained from these prior experiments at various time points over 96 h (Figure 5) Results indicate that although rates were slightly lower, using media with osmolalities 310 and 330 mOsm/kg had no significant impact on cleavage at 6 h or development of >2 cells at 30 h compared with 270 or 290 mOsm/kg. However, comparing 270 or 290 mOsm/kg, media of 310 or 330 mOsm/kg significantly decreased embryo development to ≥8 cells at 48 h, early blastocyst

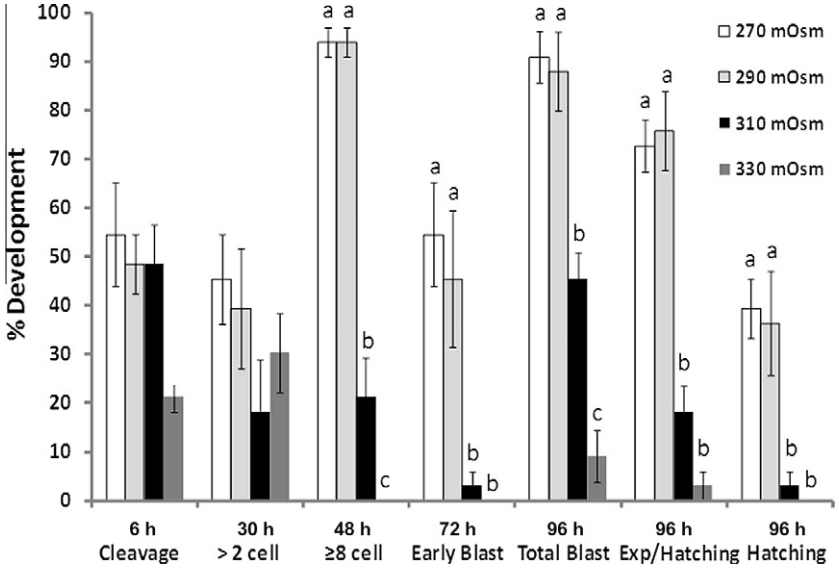

Figure 5 Development of 1-cell mouse embryos in media covering a range of osmolalities that were achieved under various microdrop preparation conditions. Negative impact of elevated osmolality on embryo development is evident as early as 48 h after culture. Different letters within a time point represent significant differences between treatments. For development to ≥8 cells at 48 h: 270 versus 310, $P < 0.0001$; 270 versus 330, $P < 0.001$; 290 versus 310, $P < 0.0001$; 290 versus 330, $P < 0.0001$; 310 versus 330, $P < 0.04$. For early blastocyst formation at 72 h: 270 versus 310, $P < 0.01$; 270 versus 330, $P < 0.01$; 290 versus 310, $P < 0.04$; 290 versus 330, $P < 0.03$. For total blastocyst formation at 96 h: 270 versus 310, $P < 0.003$; 270 versus 330, $P < 0.0001$; 290 versus 310, $P < 0.005$; 290 versus 330, $P < 0.0001$; 310 versus 330, $P < 0.01$. For hatching at 96 h: 270 versus 310, $P < 0.001$; 270 versus 330, $P < 0.0001$; 290 versus 310, $P < 0.0001$; 290 versus 330, $P < 0.0001$. Exp = Expanded.

formation at 72 h, total blastocyst formation at 96 h and hatching at 96 h. Additionally, compared to 310 mOsm/kg, 330 mOsm/kg yielded significantly lower rates of development to ≥8 cells at 48 h and total blastocyst formation at 96 h. No significant differences in embryo development were observed between embryos cultured in 310 and 330 mOsm/kg at any other time point or between 270 and 290 mOsm/kg at any time point (**Figure 5**).

Discussion

Minimizing stress imposed upon preimplantation embryos *in vitro* is crucial for optimizing their development. Proper laboratory techniques are central to this endeavour as improper approaches utilized during routine procedures in the laboratory have the ability to unintentionally and unknowingly alter the growth environment and introduce stress. This is demonstrated by examination of various common environmental conditions encountered within an IVF laboratory during preparation of culture dishes and impact on resulting media osmolality. Osmolality is a crucial media characteristic important for supporting in-vitro embryo development. As media osmolality rises above a threshold, preimplantation embryo development is compromised (Dawson and Baltz, 1997; Richards et al., 2010; Xie et al., 2007) and apoptosis is increased (Xie et al., 2007). While it has been shown that detrimental shifts in media osmolality can be obtained due to liquid absorption when using culture dishes made of improper materials, such as polydimethylsiloxane (PDMS) without the proper protective coating (Heo et al., 2007), this is the first report that demonstrates that approaches or environmental conditions present during media preparation can also impact media osmolality, causing shifts that achieve this detrimental threshold. Temperature of the working surface, volume of media and technique used to prepare microdrops can all individually result in a significant osmolality increase. Additionally, the airflow that is common to sterile work hoods can exacerbate evaporation when used in conjunction with other preparatory conditions and further increase media osmolality. Therefore, from a practical standpoint, to decrease the change of osmolality shifts during dish preparation, very detailed protocols should be implemented that consider these environmental parameters.

Importantly, the current study observed no change in media osmolality after 24 h of culture. This is not surprising considering oil overlay is used to help prevent evaporation and osmolality shift. This indicates that the period of time for microdrop preparation before oil overlay was responsible for alterations in media characteristics and should be considered as an important variable. Accordingly, number of drops per dish and number of dishes prepared before addition of oil should be closely monitored and limited. Additionally, unwashed oil was used in these experiments. It has been postulated that washed oil may be beneficial due to fluid saturation and avoidance of liquid absorption compared with unwashed oil. However, unwashed oil in this case did not appear to significantly alter osmolality through absorption of water. It is unclear whether using washed oil would have any beneficial impact on maintaining appropriate media osmolality or whether the washing solution itself may be important (water versus culture medium).

To determine whether the observed media osmolality shifts obtained under working conditions could impact subsequent embryo development, this study examined growth of F1 hybrid 1-cell mouse embryos in media with protein supplementation adjusted over the range of osmolalities obtained in this study. Using the formulation of HTF (Quinn et al., 1985), a common commercial human IVF medium, media were formulated with osmolalities ranging from ~270 mOsm up to ~330 mOsm/kg by adjusting NaCl concentration. Although an increase in NaCl concentration was reported to influence embryo development in early embryo studies, it was later shown that the impact was due to an osmolality increase, rather than NaCl concentration (Dawson and Baltz, 1997). Therefore, observed impacts on embryo development in the current study are likely limited to osmolality. However, it should be mentioned that, in addition to osmolality increase, evaporation of media during dish preparation can impact concentration of other components in the culture media. This may in itself also impact embryo development and is another consideration for preparing culture dishes in an appropriate fashion to avoid media evaporation. Regardless, the current findings are in agreement with other studies examining impact of osmolality on embryo development (Brinster, 1965; Dawson and Baltz, 1997; Hay-Schmidt, 1993; Liu and Foote, 1996; Miyoshi et al., 1994; Richards et al., 2010), as they demonstrate a negative impact of osmolality increase (>310 mOsm/kg) on embryo development. Importantly, the current findings use an alternative medium common in human IVF and a more robust model system than prior studies, by using F1 hybrid 1-cell mouse embryos (Hadi et al., 2005) with protein-supplemented media (most prior studies utilized CF1 mice with potassium simplex optimized medium and no protein supplementation). This further demonstrates the importance of media osmolality in the context of conditions used in the IVF laboratory. The current study has also examined additional time points during 96 h of development, rather than just blastocyst formation to increase assay sensitivity, which adds to the existing literature.

It should be noted that, although the osmolality of HTF is commonly ~280–285 mOsm/kg, this study included 270 mOsm/kg due to the use of commercial IVF media that now use lower osmolalities (~260–270 mOsm/kg). This comparison of lower osmolality is useful as it demonstrates a lower osmolality is compatible with embryo development and also provides a method to avoid harmful effects of media osmolality increases due to improper laboratory techniques during culture-dish preparation. Use of a medium with a lower osmolality may be more forgiving in regard to preparation technique and harmful osmolality rises. It should also be noted that although HTF media used in these studies is a common commercial media used in human IVF, it is devoid of amino acids. Specific amino acids, such as glycine, act as osmolytes and regulators of cell volume and protect the developing embryo against high osmolality (Baltz and Tartia, 2010; Dawson and Baltz, 1997; Dawson et al., 1998; Hammer and Baltz, 2002, 2003; Richards et al., 2010). Thus, use of media with these organic

osmolytes is recommended to protect against damaging effects of increased osmolality.

In conclusion, especially when preparing microdrops or using other small-volume approaches for embryo culture, embryologists should be aware of airflow, working-surface temperature and method of drop preparation and adjust protocols accordingly to optimize the culture system. Working expeditiously and with consistency assures repeatable laboratory conditions and prevents detrimental shifts in media characteristics such as osmolality.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 17 May 2011; refereed 6 October 2011; accepted 11 October 2011.