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Mouse embryo assay to evaluate polydimethylsiloxane (PDMS) embryo-toxicity*

F. Colucci, *Member, IEEE*, P. McKeegan, H.M. Picton, V. Pensabene, *Member, IEEE*

***Abstract* — *In vitro* embryo culture to support *In Vitro* Fertilization (IVF) procedures is a well-established but still critical technique. In the last decade first attempts to use microfluidic devices in IVF have shown positive results, enabling to control the culture conditions and to preserve the quality of the embryos during their development. In this study we completed an industry standard mouse embryo assay (MEA) to exclude potential toxic effects of PDMS.**

I. Introduction

Assisted reproduction technology (ART) concerns the procedures, interventions and therapies that are used to treat human infertility. *In vitro* fertilization (IVF), the most common and effective type of ART, consists of the removal of oocytes from the woman's ovaries and their fertilization with sperm in a laboratory. Once fertilized the developing embryo is then implanted back in the woman's uterus.

In the biomedical research community ART and IVF are widely used procedures to generate genetically altered (GA) mice and large animals as models to study the effects of gene function and regulation in human disorders and diseases. Finally, IVF is a key biotechnology used to preserve endangered animal species and to control breeding and increase progeny in agriculture.

The implantation rate following IVF (i.e. the percentage of embryos which successfully implant compared to the number of transferred embryos) varies with species, but it is in general suboptimal [3]. Specifically, for humans and for GA mice, implantation failures are often ascribed to the difficulties to control and preserve the microenvironment surrounding the embryo during its development [4].

The culture techniques used to grow mouse embryos *in vitro* have changed very little over recent decades. The most common method consists of culturing groups of embryos in Petri dishes in drops of 20 to 100 µL of defined culture medium, covered by mineral or paraffin oil to avoid evaporation. In the last decade, microfluidic miniaturized platforms have been designed and proposed as innovative systems to support IVF procedures. This new culture strategy aims to reduce the invasive handling of medium and 1 cell zygotes during the 3.5 days of preimplantation embryo development *in vitro*, to limit the amount and the consumption of medium, and to mimic the natural fluid flow that draws the zygotes through the fallopian tube to the site of implantation in the uterus. These new culture systems (discussed in Section II and summarized in Table I) have shown advantages, such as the reduction of culture medium volumes, the control of fluid with mL accuracy [5] and the compatibility with optical and fluorescent imaging. Some of these systems even support improved quality of the mouse embryos in terms of cleavage rate (defined as the number of embryos completed first cell division occurring at 24 hours), blastocyst rate (i.e number of blastocysts at 72 hours) and increased implantation rate.

Being derived using microfabrication processes, the first microfluidic devices were constructed with materials typically used in microelectronics, such as silicon, borosilicates, glass or thermoplastic materials, such as Poly(methyl methacrylate) (PMMA) [6] and polycarbonate (PC) [7]. With the advent of soft lithography [8] and rapid prototyping techniques [9], elastomers like polydimethylsiloxane (PDMS) have become the main fabrication material for prototyping of microscale devices in research and industry [7]. PDMS is known for its ease of use (simple curing process and design adaptability), optical transparency, gas permeability and biocompatibility but also for limitations and adverse effects, such as deformation, evaporation, small molecules absorption, leaching of uncross linked oligomers, and hydrophobic recovery [7].

We report in the following section some examples of PDMS-based microfluidic devices that support *in vitro* culture and development of mouse embryos. However, as result of this review, the specific toxicity of PDMS on embryo development has never been directly assessed.

In order to better evaluate the potential translation of these systems to human embryology and IVF, we evaluate here the toxicity of the PDMS by using a Mouse Embryo Assay (MEA) that represents the industrial gold standard for the evaluation of material compatibility.

II. Microfluidic Devices for Embryo Culture

Microfluidic culture systems could potentially support most parts of an ART cycle in a closed system which could reduce handling, stress and human error. Gamete selection,

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F.Colucci is with the School of Electronic and Electrical Engineering, University of Leeds, Leeds, UK (e-mail: elfc@leeds.ac.uk).

P. McKeegan and H. Picton are with the Reproduction and Early Development Research Group, Discovery and Translational Science Department, Leeds Institute of Cardiovascular and Metabolic Medicine, School of Medicine, University of Leeds, UK LS2 9JT (e-mail: P.J.McKeegan@leeds.ac.uk, H.M.Picton@leeds.ac.uk).

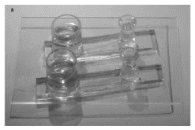
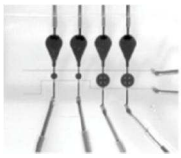
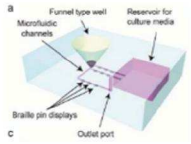
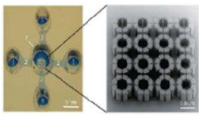
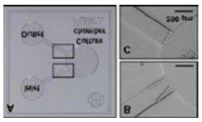
V. Pensabene is with the School of Electronic and Electrical Engineering and the School of Medicine, University of Leeds (phone: +44 (0) 113 3432070; e-mail: V.Pensabene@leeds.ac.uk)

IVF and development [10][11] have been successfully performed in microfluidic systems. "Tab.1" summarizes the key-features offered by recently developed microfluidic devices for *in vitro* embryo culture reported in the literature. Research groups working in this area have demonstrated that microfluidic devices can be used to successfully culture mouse embryos, with the final goal of applying this approach for mouse breeding or even used in human ART. The approaches used vary from embryo culture in microchannels [11][12], to embryo culture in microchambers [12]–[15], secured in place through the use of wall-like structures or valves [12][15]. Culture conditions change from device to device such that: embryos are either cultured individually [12] or in groups [12][15][16][17], and the volume of medium is reduced from the standard 20-100 µL to 0.01 to 10 µL. Cultures are performed using single step media with [13][17] and without [10][16] refreshment, in dynamic or static conditions respectively. Unfortunately, the majority of these devices still require the use of mineral oil [10][16][17] to stabilize temperature, osmolality and pH. Quality and composition of the oil have been correlated with embryo cleavage (to morula and blastocyst stages) and embryo viability [18] that are both markers of embryo developmental competence rate.

III. MOUSE EMBRYO ASSAY IN MICROFLUIDICS

The MEA is currently the gold standard bioassay used in research and industry to evaluate the toxicity of any compound or device that may be exposed to embryos. New materials, devices or culture media are screened with this test. Similarly, samples from different batches of products (e.g. plastic dishes, flask, or pipettes) are regularly tested after manufacture. The MEA is thus designed to ensure consistent quality, reliability and safety of products (media or devices) to be used in assisted conception clinics and it represents the

TABLE I. EXAMPLES OF MICROFLUIDIC DEVICES FOR *IN VITRO* CULTURE OF MOUSE EMBRYOS

					
Research group	Raty et al., 2004 [16]	Melin et al., 2009 [15]	Heo et al., 2010 [17]	Ma et al., 2011 [10]	Esteves et al., 2013 [12]
Culture environment					
Medium	M16	HTF supplemented with 10% serum replacement	KSOM + ½ AA with D-Glucose and phenol red Cat # MR 121-D	KSOM	α-MEM + 0.2% w/v BSA + 50µg mL^{-1} gentamicin sulphate
Incubator conditions	37 °C, 5% CO_2	37 °C, 5% CO_2, 5% O_2, 90% N_2	37 °C, 5% CO_2 and 20% O_2	37 °C, 5% CO_2	37 °C, 5% CO_2
Single vs. group culture	Group culture (10 embryos)	Group culture (2 embryo/chamber)	Group culture (13 – 15 embryos)	4x4 array of single embryos	Single and group culture (5 and 20 embryos)
Device characteristics					
Volume	10 µl	0.1 µl	10 µl	0.5 – 10 µl	0.030 and 0.270 µl
Material	Silicon/borosilicate PDMS/borosilicate	PDMS	PDMS-Parylene-PDMS	PDMS	PDMS
Retrieval	Yes	No	Yes	Yes	Yes
Culture characteristic					
Refreshed medium	No	No	Yes	No	Yes
Mineral oil	Yes	Yes	Yes	Yes	No
Static vs. dynamic culture	Static	Static	Pulsatile, peristaltic pumping	Static	Passive pumping

first stepping stone for the adoption of a new product (i.e. a microfluidic PDMS device) into this field.

The standard MEA consists of monitoring the growth of mouse embryos in contact with the material or device "under test" from 1 or 2 pluripotent cells stage to the blastocyst stage and in assessing embryo morphology and viability at different time points. Protocols for this qualitative and quantitative assessment can vary from company to company (e.g. Vitrolife, Charles River) depending on the complexity, novelty and stability of the product to be evaluated, the selection of mouse strain, the number of replicates and/or the number of analysed end points are usually not consistent between companies.

IV. MATERIALS AND METHODS

A. *Device fabrication*

Microfluidic devices were fabricated in PDMS (Sylgard® 184, Down Corning, MI, USA) using the standard soft lithography technique [8]. This process included the fabrication of SU-8 2050 and SU-8 2035 molds by lithography to form two PDMS layers presenting a 400 nL circular chamber, accessible by two 7.5 mm long lateral channels. The two layers were assembled using oxygen plasma treatment (600 mT, 100 W, 40 s). This process allowed for contamination removal (chemical), oxidation and activation of the surfaces. Once assembled, the devices were immediately filled with embryo tested water using disposable, sterile syringes and stored at 4 °C until use to preserve hydrophilicity. Before loading embryos and cell culture medium, the devices were sterilized by exposure to UV light for 30 min.

B. *Murine embryo production*

Murine cryopreserved presumptive 1 cell zygotes were kindly provided by the Mary Lyon Centre (MRC Harwell Institute, Oxford, UK). These were obtained from mature murine metaphase II oocyte-cumulus-complexes retrieved from C57BL/6NTac mice after superovulation and IVF. Straws containing frozen 1 cell zygotes were held in air for 30 s, and plunged into room temperature water until the contents had visibly thawed. The straws were cut at the seal and the plug bisected to push the contents into a 60 mm, hydrophobic culture dish. Embryos were incubated for 5 min and washed 2 times in 100 µl drops of M2 medium at 37°C. Embryos were then washed 3 times in 10 µL microdrops of pre-equilibrated potassium-supplemented simplex optimized medium (KSOM) under oil before culture.

The experimental procedures involving animals use described in this paper were approved by the Institutional Animal Care and Ethics Committee.

D. *Murine embryo culture in microfluidic devices*

The microfluidic device was placed inside a 60 mm MEA tested culture dish and surrounded with 4 mL of embryo-tested sterile water. Devices were prepared by flashing the microfluidic chamber with KSOM for 10 times. 10 µL drops of fresh KSOM were added to inlet and outlet before overnight pre-equilibration at 37°C (5% CO_2, 5% O_2 in a humidified nitrogen atmosphere). 9 embryos were then placed in a 10 µL drop in the inlet port of the device and loaded by using an EZ-grip embryo handling pipette with a 135 µm diameter tip. Media was then drawn through from the outlet port until all embryos entered the central chamber. 10 µL drops of pre-equilibrated KSOM were then added to channel inlet and outlet before culture at 37°C under 5% CO_2 in 5% O_2 in humidified nitrogen in a MINC bench top incubator (Fig. 1). Embryo loading was equally successful and practical using a range of bulb and pipettor embryo handling devices common to clinical and research ART laboratories. Embryo cleavage rates were assessed after 24 h and blastocyst rates were assessed after 120 h of continuous culture. Embryos were examined under a Nikon stereomicroscope with Tokai Hit heated stage set to 37 ºC. Examples of early cleavage (2-8 cell) and blastocyst stage embryos are shown in Fig. 2.

Figure 1. Experimental set up – Embryo loaded microfludic devices placed within a 60 mm tested culture dish, in a MINC bench top incubator.

Figure 2. Examples of 2-cell (left) and blastocyst stage (right) embryos cultured in microfluidic devices (scale bar 50 µm).

V. RESULTS AND DISCUSSION

A. *Mouse embryo assay*

Following industrial standard protocols (www.criver.com), our MEA test compared cleavage and blastocyst rates (Fig. 3) resulting from 120 h cultures of groups of 9 embryos cultured in microdrops (1 µL KSOM per embryo) or in PDMS devices. No significant differences were found between microdrop and microfluidic device cultures in terms of cleavage rate (94.7% ± 1.6, n=117 embryos across 13 microdrop cultures vs 94.4% ± 2.5, n=135 embryos across 15 device cultures) and blastocyst rate (94.1% ± 1.8 vs 94.3% ± 2.3 respectively).

Microfluidic culture had no effect on embryo development rates ($p = 0.93$), and based on this standard test

for embryo toxicity, PDMS was shown to be non-toxic and not to affect embryo survival (Fig. 2).

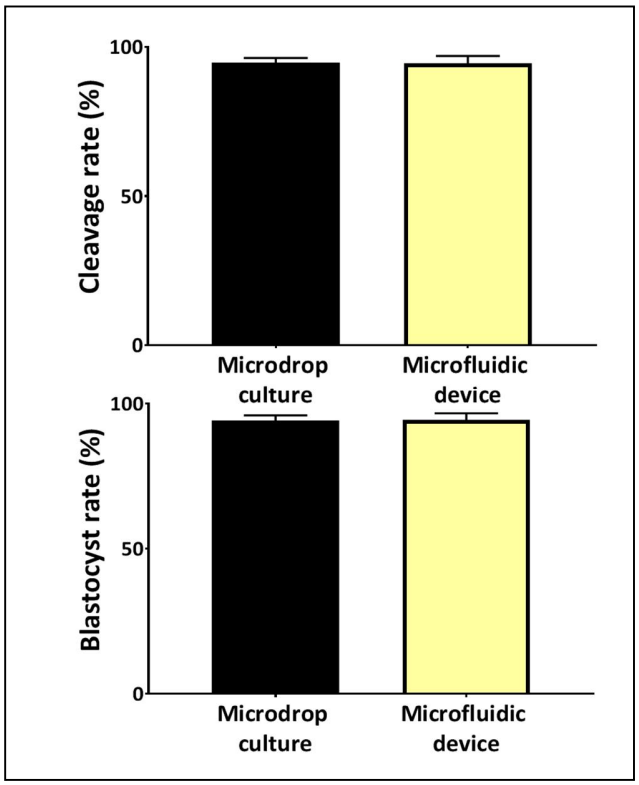


Figure 3. MEA – Cleavage rate and Blastocyst rate Microdrop: n=117 embryos across 13 microdrop cultures. Device: n=135 embryos across 15 device cultures.

VI. Conclusion

In this study, we evaluated the material toxicity of PDMS on embryo development, by using an industry standard MEA. PDMS has so far been assumed to be a biocompatible plastic for *in vitro* embryo culture, based on its general biocompatibility and the positive results obtained by culturing zygotes in microfluidic devices. Our tests, which were designed to uniquely observe the PDMS compatibility, independently from the device characteristics and functions, excluded detrimental effects of the material on the embryo quality. Even if the MEA test represents the standard step for the introduction of novel materials or devices into the clinical IVF usage, we believe that subjective morphological assessment of mouse embryos represents a poor indicator of the developmental competence of the embryos. A complete and safe screening method should assess the potential effects or alterations induced by the new methods/materials/devices on the genetic, epigenetic and metabolic health characteristics of the embryos *in vitro* and/or on their subsequent implantation potential.

Acknowledgment

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