Comparable Results between Continuous and Sequential Media on Mouse Embryo Cultured in Time-Lapse Incubator

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Abstract. Time lapse incubator now became a new technology for clinical In Vitro Fertilization. This incubator allows embryo observation continuously and periodically without taking the embryo out from the incubator. The nature function of time lapse incubator requires continuous incubation without taking out the embryo from the incubator, means there is no change over media during incubation. In the other hand most culture media that available in the market distinguish between cleavage and blastocyst stage embryo. This known as sequential media. This experiment compared the use of continuous and sequential media during in vitro embryo culture using time lapse incubator. One cell mouse embryo derived from F1 (C57BL/J MARP x CBA/MARP) were used in this experiment. Embryos were culture for 5 days until they reach blastocyst stage. The continuous media (Global Media, Life Global) was used to culture media from day 1 till day 5, while sequential media were divided into two parts. Cleavage media (SIVF-Cleavage, Cook Medical, Brisbane) was used from day 1 till day 3, and Blastocyst media (SIVF-Blastocyst, Cook Medical, Brisbane) was used from day 3 till day 5. Control embryos were cultured in sequential media (SIVF- Cleavage and SIVF-Blastocyst, Cook Medical) in bench top incubator (MINC, Cook, Brisbane). A total of 320 one cell embryos were used in this experiment. Embryo development was evaluated by the number of embryo developed into blastocyst.

Keywords:

1. Introduction

In Vitro fertilization is a new emerging methods to help infertile couple to fulfil their dream to bear their own child, but also to help farmers to get a high quality farm animals, pet lovers and conserving endangered animal. The birth of Louise Brown, the world's first 'test-tube baby', has come to signify the moment at which technologically assisted human reproduction became a reality [1]. On the other hand similar age has also been reach by development IVF in cattle [2]. Both human and animal IVF are known to play an important role either a s a model for human clinical IVF or for animal breeding and conservation. Porcine [3][4], fish [5], dog [6], rabbit [7], frog [8], non-human primate [9], tiger [10-12] and some other animals has also been used to develop model for clinical IVF or for them self as a conservation method.
Maintain optimal in vitro culture is important to get the best outcome in IVF. In Vitro embryo culture system has been developed for more than 50 years [13]. Culture condition involves defined chemical composition [14], osmolarity [14-17], temperature [14][18][19], pH [14][15][20], gas atmospheric [14], and light [21][22]. Any disruption on embryo culture is a potential factor that may impair embryo development.

Recently, time lapse became the latest technology in in vitro embryo culture. Numerous studies have been published that confirmed the safety of time-lapse technology [23]. Time lapse allows continuous embryo observation every 5 to 20 minutes without taking the embryo out from the incubator. This method will eliminate disruption due to taking the embryo out from the incubator for microscopic observation. Also more observation can be done on the embryo compare to the current observation that usually done every 24 hours. It means more information can be obtained from time lapse data that allows the scientist to select the best embryo according the images obtained from the time lapse. Further development of time lapse is annotation. Trained artificial intelligence system allows the software to help analyse the embryo according to their development pattern [24-28].

Time lapse technology allows embryo to be cultures from day 1 until day 5 without any disruption. It means there is no media change over as traditionally proposed in sequential media [29-31]. During in vivo or in vitro development, embryo requires different media composition to support it specific stages. [30]. The development time lapse media, which allows embryos stay in the same culture drop from day 1 until day 5 is a new system that needs modification in some of component in embryo culture media[32-35]. This paper will compare the use of sequential media vs. single step media on mouse embryos cultured in time lapse incubator.

2. Methodology

Animals
Mouse (F1: C57BL/J MARP x CBA/MARP) embryos were used in this experiment. Female mouse was super ovulated by intraperitoneal injection of 5iu PMSG (Folligon, MSD-Intervet, Bendigo) at 4 pm, then 48 hours later was injected with 5iu hCG (Chorulon, MSD-Intervet, Bendigo), followed mating with proven male F1 stud. Upon the finding of vaginal plug on the next day, the mouse was sacrificed at 10 am (18 hrs post hCG). One cell mouse embryos were collected from ampulla in a home-made KSOM-Hepes media, then cumulus cells were removed using 100 iu Hyaluronidase (H-3884, Merck-Sigma Aldrich, Castle Hill, NSW). All one cell embryos were pooled then randomly distributed into two different media treatments (One-step and sequential) the cultured in time lapse incubator (MIRI-TL, ESCO Singapore) and control in sequential media in MINC (Cook Medical, Brisbane).

Embryos in time lapse incubator were culture in culture coin (ESCO, Singapore), while control embryos were cultured in 60 mm dish (Falcon, BD, Noble-Park).

Embryo culture protocol
Culture coins: Each well of culture coins filled with 20 microliter media then covered under mineral oil (M-1840, Merck-Sigma Aldrich, Castle Hill, NSW), while, 60 mm dish: a drop of 30 microliter was made in the dish, 5 to 6 drops per dish covered under oil. All dishes were equilibrated overnight in an incubator (HeraCell VIOS 150, Thermo Fisher, Mulgrave VIC) in 37 degree C, 5% CO2 with humidified air.

One embryo was placed in each well of culture coin and up to 5 embryos were placed in each drop in control group. Control group embryos were checked every 24 hour for their in vitro development. While embryos in time lapse were recorded every 5 minutes at 7 different focal plane.

Embryos cultured in one step media were left in culture from day 1 until day 5, while embryo in sequential media were cultured in cleavage media for 72 hours (3 days) then transferred to Blastocyst media until day 5 (120 hrs). Control embryos in MINC incubator also changed over to Blastocyst media at 72 hrs. Any control group embryos which fail to develop according its timeframe or milestone will be removed from the drop.
On Day 5 (120 hrs) all embryos were analysed for their blastocyst formation. Any embryo develop to blastocyst will be classified as blastocyst regardless their advancement ie, Expanded, Hatching or Hatched blastocyst.

There are five replicates with total 320 embryos were used in this experiment. Data were analysed using Chi-square for number of embryos develop in each stage and Anova for timeline on each milestone embryo development. All animal used in this experiment were approved under Monash University Animal Ethics MMCA 2015/57.

3. Result and Discussion

During in vivo development, mouse embryos reach blastocyst stage approximately at 3.5 day in culture [36]. The study showed that most of mouse embryos develop their blastocoel at 96 hrs post hCG injection, however some of them have blastocoel formation at 90 hrs post hCG. This indicated that during that time period the embryo already reach uterine cavity [36]. A total of 320 embryos were used in this experiment with almost equal number in each treatment and control. Those embryos were divided into 5 replicates. Observation using time lapse on embryos cultured in both single step and sequential media also in the control group showed that all embryos entering early blastocyst stage at 90-96 hrs, and forming fully blastocyst from 96 to 102 hours. While control embryos in MINC showed fully blastocyst on day 4 or 96 hours in culture.

| Table 1. Number of mouse embryos used in each treatment and replicate |
|---------------------------------------------------------------|
| Treatment          | 1   | 2   | 3   | 4   | 5   | total |
| Single Step        | 20  | 20  | 20  | 22  | 25  | 107   |
| Sequential         | 20  | 20  | 20  | 22  | 25  | 107   |
| Control            | 20  | 20  | 20  | 21  | 25  | 106   |
| Total              | 60  | 60  | 60  | 60  | 60  | 320   |

During 4 days in culture most embryos form blastocyst in either control, single step or sequential media. Those percentage indicated embryos capability to develop normal in vitro in respective media and treatment. Statistical analysis showed no difference (P<0.01) on number and proportion on embryos develop from one cell to 2-cell, Morula and Blastocyst (Table 2). These results indicated that using sequential or single step media did not affect proportion on embryo develop in vitro.

| Table 2. Number embryos developed to 2-cell, Morula and Blastocyst |
|---------------------------------------------------------------|
| Treatment          | Number | 2-cells | Morula      | Blastocyst   |
| Single Step        | 107    | 100 (93.46%) | 98 (91.59%) | 93 (86.91%)  |
| Sequential         | 107    | 101 (94.39%) | 98 (91.59%) | 94 (87.85%)  |
| Control            | 106    | 100 (94.33%) | 96 (90.56%) | 92 (86.79%)  |

Further observation on time lapse incubator showed that the time line on embryo development between single step and sequential media did not show any difference (P<0.01). Observation on timeline show more accurate number or result compare to control group embryos where observation was made every 24 hour (Table 3). However overall observation showed that timeframe development between Single Step, Sequential and Control are remains within normal embryo development. Those timeframe in line with observation made by Wale and Gardner [14] using mouse model. Meanwhile typical human embryos will reach blastocyst stage at day 5 or after 120 hours in culture [14]. On the other hand mouse embryo development to blastocyst is 24 hours earlier than human or mouse embryos reach blastocyst at d3.5 or d4 [36].
Table 3. Time line from cell collection to 2-cell, Morula and Blastocyst

| Treatment     | 2-cells (hrs) | Morula (hrs) | Blastocyst (hrs) |
|---------------|---------------|--------------|------------------|
| Single Step   | 22±2.15       | 68±3.10      | 94±2.55          |
| Sequential    | 22±2.20       | 67±2.95      | 92±2.21          |
| Control       | 1             | 3            | 4                |

*) change over at 72 hours. 10 minutes required for change over.

Results in table 3 showed no difference (P<0.01) on time frame between Single Step and Sequential media on their time different from 1 cell to 2-cell, Morula and Blastocyst.

4. Conclusion

The results showed that both Single Step and Sequential media are comparable to traditional group embryo culture on MINC using sequential media. There is no difference between Single steps and sequential media on development proportion and time frame. However if we look at the time spent and activity, culture using single step media will be more desirable due to the minimum disturbance during in vitro culture.

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