Chapter

Effects of Different Types of Incubators on Embryo Development and Clinical Outcomes

Ji Liu, Yan-Hua Zhou, Xiao-Xiao Wang, Ling-Xi Tong, Yan-Hong Li, Ling Liu, Zhi-Yan Xu and Hong-Hui Wang

Abstract

Main differences of incubators are humidity, temperature and gas control ways, which play important roles in regulating the steady state of culture media. In this study, we compared the effects of different types of incubators (air jacket incubators and water jacket incubators) on embryo development and clinical outcomes in human assisted reproduction. We found that temperature recovery time in air jacket incubators was significantly shorter than that in water jacket incubators. Although the O_2 recovering time was also significantly shorter in air jacket incubators as compared with the water jacket incubator, no significant differences were observed in CO_2 recovering time between two groups, which was also verified by pH recovering time of culture media. Besides, the temperature of culture medium in the dish covered with oil recovered more quickly in the air jacket incubators. However, there were no significant differences observed in the fertilization rate, Day 3 high-quality embryo rate, blastocyst rate, good blastocyst rate and clinical outcomes between two groups. These results indicate that the microenvironment, especially the temperature, in air jacket incubator recover faster than that in water jacket incubators, however, there were no significant differences in embryo development and clinical outcomes between two types of incubators.

Keywords: Embryo culture, Incubator, Temperature, CO2

1. Introduction

For most kinds of cell culture, a suitable culture condition depending on the cell type is used. Human embryos are usually cultured in incubators in a humidified condition with 5–7% CO2 with or without reduced oxygen at 37°C condition. Although culture media are very important for embryo development, the environment of embryo culture is also a critical factor, which provides stable conditions for embryo development by controlling pH and temperature of the culture media.

During oocyte fertilization and embryo development, maintaining an appropriate and stable culture environment for gametes and embryos is the guarantee for protecting the developmental competence of embryos. The primary functions of

incubators are to maintain stable temperature, optimal pH levels for embryo growth and stable osmolality of the media [1, 2]. Different types of incubators based on these principles have been developed in the past few years, which include water jacket incubators, benchtop incubators, drawer incubators and time-lapse incubators [3]. The main differences of these incubators are temperature control system, gas control system (the gas premixed incubator, the conventional gas incubator with CO2 only) and humidity. For the air jacket incubator, the gas is pre-heated. While the warming process of culture medium in the water jacket incubator is thermal conductivity by gas, which is heated by the water jacket.

As for embryo culture, the culture medium is usually covered by mineral oil for maintaining a normal range of osmolarity for embryo development. Besides, with the development of CO2 sensors, conventional thermal conductivity sensors were replaced by infrared sensors [3]. Therefore, the dry incubator becomes a new choice for embryo culture. Compared with the conventional incubator, the dry incubator is easier to clean due to its smaller size and the risk of contamination is also reduced significantly under the moisture-free environment [4].

Although dry incubators have been widely applied to the field of human IVF, their effects, especially the clinical outcomes, are reported rarely. It was reported that the early stage embryo and blastocyst formation rates in top-load mini-incubators are superior to that in front-load conventional incubators [5]. However, Mohamed Fawzy and colleagues reported that embryos cultured in dry incubators showed significantly decreased implantation and clinical ongoing pregnancy rates [6]. However, in their study, live-birth rate was not reported and the paired comparison for embryos derived from the same patient was also of lack.

In the present study, we compared the effects of the air jacket incubator and conventional water jacket incubator on embryo development and the final clinical outcomes. We found that the temperature and gas concentration in air jacket incubators recovered more quickly than conventional water jacket incubators, but there were no significant differences observed for embryo development and clinical outcomes.

2. Materials and methods

2.1 Patients

Ethical approval was obtained from the Medical Ethics Committee of Weihai Maternal and Child Health Hospital (WFEY-QR-CR-825, 3 January 2017). The written cognitive and approval consents were also signed by patients. Patients undergoing routine IVF treatment (the number of COCs acquired ≥10) at the Reproductive Medical Center of Weihai Second Municipal Hospital between Jun 2017 and Aug 2019 were treated as candidates for this study. The characteristics of patients were listed in **Table 1**, including age, body mass index (BMI), basal sex hormone levels, duration of infertility. Women with endometriosis, poor endometrium (<8 mm diameter), premature ovarian insufficiency on the hCG trigger day or the transfer day were excluded. Samples from their husbands were also excluded if they had severe asthenospermia/oligospermia and aspermia.

The follicles of women receiving gonadotropin releasing hormone agonist (GnRH-a) long protocol were monitored by ultrasound. When 10 or more follicles had reached a mean diameter of ≥14 mm, the women were given appropriate dose of hCG to induce oocyte meiotic maturation. Cycles with more than 10 COCs retrieved were assigned in this study and all COCs were allocated equally and non-selectively to either incubator.

Age	30.88 ± 0.57
BMI(kg/m2)	23.91 ± 0.51
Basal E2	297.2 ± 69.45
Basal FSH	6.53 ± 0.46
Duration of infertility (y)	5.4 ± 0.62
Antral follicle count(≥14 mm)	10 ± 2.22
No. of oocytes collected	23.56 ± 1.05
No. of matured oocytes	18.6 ± 0.65
No. of embryo transferred	1.63 ± 0.08

Note: data showed mean ± SEM.

Table 1.
Characteristics of patients in this study.

To avoid frequent opening/closing of the incubator door, the 'one patient one incubator' strategy was conducted in our study, which means COCs or embryos from one patient were cultured separately in one incubator.

2.2 Incubators and parameters monitoring

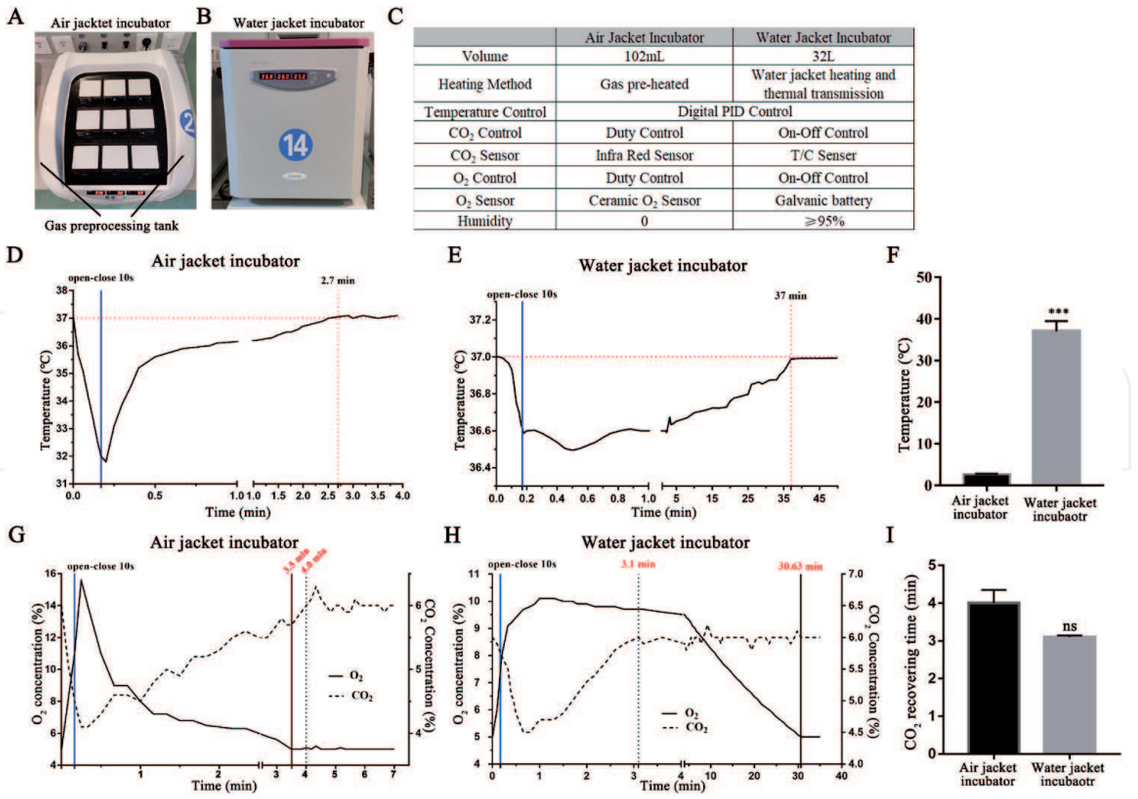
As showed in **Figure 1A** and **B**, the air jacket incubator (EC9 triple gas bench-top incubator, ASTEC CO., LTD. Japan) and a conventional water jacket incubator (Penguin AQ series/APM30D, triple gas incubator, ASTEC CO., LTD. Japan) were used in our research. The specifications of different incubators were listed in **Figure 1C**. A range of 12–15 repeated opening/closing processes were conducted in a single ART cycle (from oocyte collection to blastocyst transfer).

For temperature monitoring, a handheld temperature measuring equipment with a long and soft linear sensor (TES 1310 TYPE-K, China) was used to monitor the variation of temperature in a center-well organ culture dish (FALCON, 353037) with 1 ml medium covered with 1 ml mineral oil in the dishes. Briefly, according to the length of time consuming in routine embryo culture, we made a single 10-seconds door opening/closing process, after which the temperature of incubator chambers was detected. It should be noted that 5-seconds door opening/closing was enough for air jacket incubator, in which only one dish was usually placed. Considering the consistency of this study, 10-seconds opening/closing treatment was accepted for two kinds of incubators. CO2 and O2 recovering times were recorded according to the corresponding display panels.

For pH measurement, 5 ml medium was poured into a tube and equilibrated for overnight. At the second day, we tested the initial pH values (initial state) by a pH meter (PB-10 Sartorius). As temperature monitoring, after a 3 min holding on the thermostatic desk, pH values were recorded again (out for 3 min), after which the medium was put back into incubators and detected at 10 min, 30 min and 1 hour (showed 'in for 10 min', 'in for 30 min' and 'in for 1h' respectively).

2.3 Sperm preparation, fertilization and embryo culture

After semen liquefaction (nearly 30 minutes), density gradient centrifugation combined with swim-up was used to sort sperm with normal morphology and high motility [7]. G-IVF (vitrolife) was used to wash sperm and120,000 motile sperm/ml was used for short-time in vitro fertilization. After 4 hours co-culture, oocyte denudation was performed using mechanical method and the remaining



	Air Jacket Incubator	Water Jacket Incubator
Volume	102mL	32L
Heating Method	Gas pre-heated	Water jacket heating and thermal transmission
Temperature Control	Digital PID Control	
CO_2 Control	Duty Control	On-Off Control
CO_2 Sensor	Infra Red Sensor	T/C Senser
O_2 Control	Duty Control	On-Off Control
O_2 Sensor	Ceramic O_2 Sensor	Galvanic battery
Humidity	0	⩾95%

Figure 1.
*Comparison of the air jacket incubator and water jacket incubator. (A) and (B): Photograph of the air jacket incubator (A) and the water jacket incubator (B). (C): Physical parameters of different types of incubators. (D) and (E): Incubator temperature recovering process after a 10 seconds opening/closing process. The solid line and right vertical axis represented the changing process of the air jacket incubator. The dotted line and left vertical axis represented the change of temperature in the water jacket incubator. (F): Statistical data of temperature in different incubators. ***: P < 0.001. (G) and (H): CO2 and O2 recovering process in different incubators (G was the air jacket incubator; H was the water jacket incubator). (I): CO2 recovering time in different incubators. Ns: no significant differences.*

sperm was also removed. Depending on the presence of the second polar body, we judged if oocytes were fertilized and only these oocytes with two polar bodies were then transferred into cleavage culture medium (G1 medium, vitrolife). At day 1 (16–18 hours after fertilization), the number of pronucleus (PN) was recorded and 2PN-gametes were identified as normal fertilization, after which these gametes were transferred into new G1 medium. At day 3 and day 5, embryos were transferred into G2 medium (vitrolife) for blastocyst culture.

2.4 Embryo and blastocyst scoring

Embryos and blastocysts were graded according to the Istanbul consensus and Gardner criteria [8, 9]. Briefly, embryos (day 3) with 7–9 cells, less than 10% fragmentation by volume and symmetric blastomeres were identified the good. Blastocysts (day 5) graded 4BB or even better were identified the good, including 4BB, 4AB, 4BA and 4AA. Embryos on day 3 or blastocysts on day 5 were assessed by three experienced embryologists and the assessments were recorded individually. Although most of the results were consistent between embryologists, the lowest score (when exist) was accepted.

2.5 Embryo transfer and clinical follow-up

Using abdominal ultrasound guidance, one or two embryos (fresh or frozen–thawed embryos) were transferred to each woman. In some situations, such as the

patient with ovarian hyper-stimulation syndrome (OHSS), embryos were cryopreserved and the frozen–thawed embryos were transferred later. Serum β-hCG levels were monitored on day 14 after embryo transfer, which was used to confirm biochemical pregnancy. When the gestational sac (should have heartbeat) was observed using ultrasound one month after embryo transfer, clinical pregnancy was confirmed. Considering the possibility of failure in one cycle, we calculated the successful rate of every transfer cycles to compare the clinical outcomes in two groups. For example, if one patient was conducted two times of frozen–thawed embryo transfer (FET) (all embryos from the air jacket incubator) and was verified pregnant at last, the clinical pregnancy rate would be 50%.

2.6 Statistical analysis

Statistical analysis was performed using Student's t-test or Fisher's exact test with GraphPad Prism 7 software. Data were expressed as mean ± SEM. As for comparing the proportion of pregnancies, dichotomous outcomes data were showed as frequency and percentage. The differences between two groups were represented by computing the odds ratio with 95% confidence interval, and Fisher's exact tests was used. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Microenvironment in air jacket incubators recovers faster than that in water jacket incubators

As shown in **Figure 1A** and **B** the gas preprocessing tank takes up a large space of the incubator, which is used to heat and mix the gas (6% CO2, 5% O2, and 89% N2). After that, the warmed and mixed gas was released into culture chamber. The volume, heating method, gas control system and humidity are main differences between two kinds of incubators (**Figure 1C**). Besides, the gas control system of air jacket incubators is duty control, which provide better fault-tolerant capabilities than the on–off control system (**Figure 1C**).

During embryo culture, the door of an incubator was opened and closed frequently. We monitored the temperature recovering process after a 10s-opening/closing procedure. As shown in **Figure 1D**, after an opening-10s-closing procedure, the chamber temperature of air jacket incubators decreased steeply (the lowest temperature was 31.8°C). However, in less than 3 min (2.7 ± 0.12 min, n = 4), the chamber temperature of air jacket incubators recovered to the normal. On the contrary, despite the gentle decline of temperature in water jacket incubators (the lowest temperature was 36.5°C), it taken nearly 37 min to recover its intra-environment temperature (37 ± 2.48 min, n = 4; $P < 0.0001$) (**Figure 1E** and **F**). For gas recovering process, as shown in **Figure 1G** and **H**, CO2 recovering time was 4 min and O2 recovering time was 3.5 min in air jacket incubators. However, O2 recovering time was more than 30 min in water jacket incubators. There was no significant difference in the CO2 recovering time between two types of incubators (air jacket incubator vs. water jacket incubator: 4 ± 0.35 min vs. 3.1 ± 0.15 min, n = 4; $P > 0.5$)) (**Figure 1I**).

The temperature of culture medium in air jacket incubators recovers faster than in water jacket incubators, but there are no differences in pH values between two groups.

We also detected the change of medium temperature over time. Simulating the observation and operation of embryos, we taken out the dishes from the incubator and placed on a 37°C thermostatic desk for 3 min (**Figure 2A**). As shown in

Figure 2B, we found that the temperature of medium covered with mineral oil recovered to 37°C within 31.5 min in air jacket incubators. However, more than 65 min were taken for temperature recovering in the conventional water jacket incubator. As shown in **Figure 2C**, the pH values of G-IVF, G1 and G2 (Vitrolife) represented similar changing trends between two groups and no significant differences were observed.

3.2 There are no differences in short time IVF and embryo development between two types of incubators

Since the differences in intra-incubator micro-environment regulating method between two types of incubators, we investigated if the fertilization and embryo development were affected due to the factors of culture environment fluctuating. As shown in **Table 2**, there were no differences in fertilization rate between two groups (water jacket incubator group: 77.84 ± 2.15%, n = 498 vs. air jacket incubator group: 74.57 ± 2.24%, n = 478; $P > 0.05$). The normal fertilization rates (2PN rates) at day 1 were also similar between two groups (water jacket incubator group: 53.99 ± 3.04%, n = 498 vs. air jacket incubator group: 52.18 ± 2.74%, n = 478; $P > 0.05$) (**Table 2**). Meanwhile, there were also no significant statistical differences in the abnormal fertilization rates (≥3PN for IVF, 1 PN or ≥ 3PN for ICSI) between two groups (water jacket incubator group: 8.04 ± 1.65%, n = 498 vs. air jacket incubator group: 7 ± 1.77%, n = 478; $P > 0.05$).

On day 3, we assessed the quality of embryos in two groups and found that there were also no differences in good quality of embryos that with 7–9 symmetric blastomeres and less than 10% fragmentation by volume (water jacket incubator

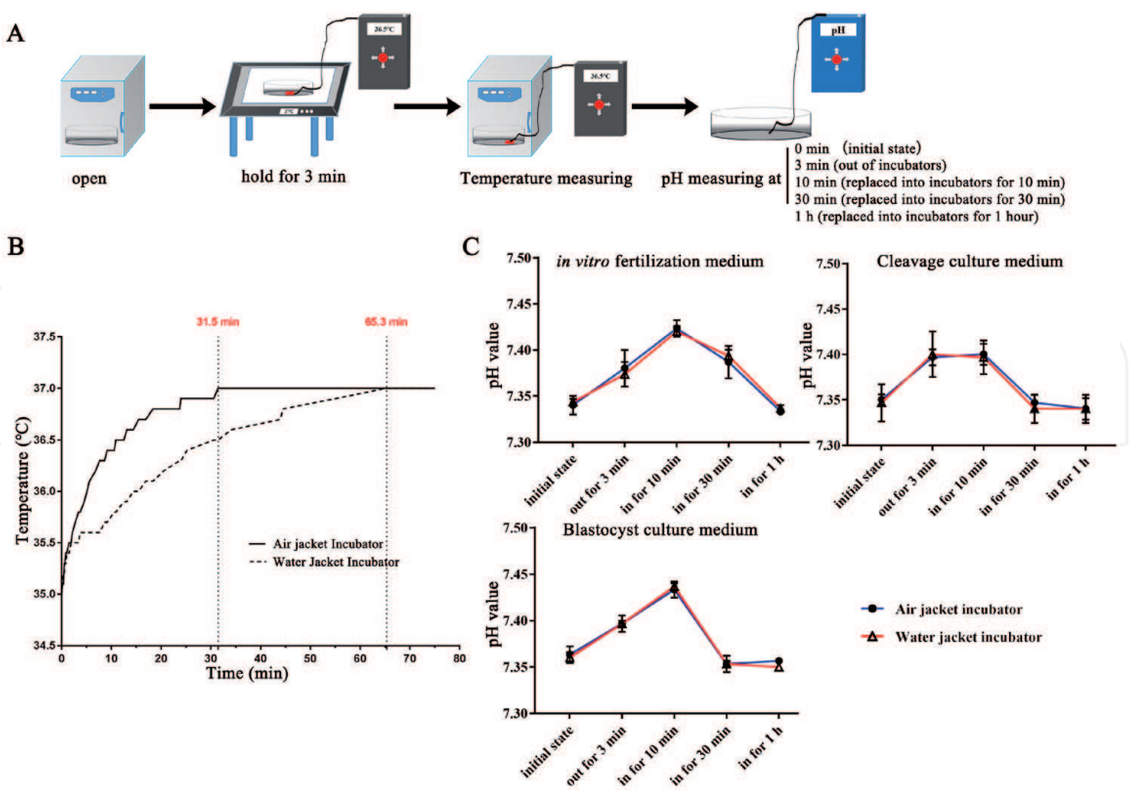


Figure 2.
Temperatures and pH values of culture medium recovering process in different incubators. (A): The cartoon indicating the timing of temperature and pH monitoring. (B): Temperature variation of culture medium covered with oil in incubators after a 3-min-handling outside the incubator. The solid line represented the changing process of air jacket incubators, the dotted line represented the water jacket incubator. (C): pH values of G-IVF, G1 and G2 recovering processes in different types of incubators.

	Air jacket incubator	Water jacket incubator	P value
Fertilization rate	74.57 ± 2.24% (n = 478)	77.84 ± 2.15% (n = 498)	0.14
Normal fertilization rate	67.57 ± 2.61% (n = 478)	69.8 ± 2.51% (n = 498)	0.47
Abnormal fertilization rate	7 ± 1.77% (n = 478)	8.04 ± 1.65% (n = 498)	0.61
D3 good embryo rate	49.48 ± 4.18% (n = 315)	50.97 ± 3.32% (n = 336)	0.7
Blastocyst formation rate	60.74 ± 3.82% (n = 269)	65.54 ± 3.97% (n = 288)	0.33
Good blastocyst rate	43.97 ± 4.92% (n = 166)	48.14 ± 4.67% (n = 191)	0.52

Note: Data presented as mean ± SEM (n). Paired t test was used for the statistical analysis between two groups. P values >0.05 indicated there were no differences between two groups.

Table 2.
Fertilization and embryo development in different types of incubators.

	Air jacket incubator	Water jacket incubator	Odds ratio (95% Cl)	P value
Biochemical pregnancy	14/26(53.85%)	22/33(66.67%)	1.71(0.62–4.92)	0.42
Clinical pregnancy	11/26(42.31%)	18/33(54.55%)	1.64(0.58–4.52)	0.43
Implantation	15/37(40.54%)	20/46(43.48%)	1.13(0.46–2.63)	0.83
Live birth	9/26(34.62%)	11/33(33.33%)	0.94(0.32–2.92)	>0.99

Note: Data presented as proportions, n(%). Fisher's exact test was used for between-group data comparisons. Odds ratio with 95% confidence interval (Cl) were also listed. P values>0.05 indicated there were no differences between two groups.

Table 3.
Clinical outcome comparisons between two types of incubators.

group: 50.97 ± 3.32%, n = 336 vs. air jacket incubator group: 49.48 ± 4.18%, n = 315; P > 0.05) (**Table 2**). As for blastocyst formation rate, the water jacket incubator group was 65.54 ± 3.97% (n = 288), compared to 60.74 ± 3.82% (n = 269) blastocyst formation of the air jacket incubator group (P > 0.05). There were no significant differences observed in good blastocyst formation (water jacket incubator group: 48.14 ± 4.67%, n = 191 vs. air jacket incubator group: 43.97 ± 4.92%, n = 166; n = 43, P > 0.05). All these results indicated that intra-incubator microenvironment regulating method does not affect fertilization and embryo development.

3.3 The clinical outcomes were also similar between two groups

As shown in **Table 3**, the rates of biochemical pregnancy and clinical pregnancy were 53.85% and 42.31% respectively in air jacket incubators, which were similar as that in water jacket incubator (rates of biochemical pregnancy and clinical pregnancy were 66.67% and 54.55% respectively). No statistically significant differences were observed between two groups (P > 0.05). The implantation rate and live-birth rate of the air jacket incubator were 40.54% and 34.62% respectively, which were also similar with that in the water jacket incubator (the implantation rate was 43.48% and the live-birth rate was 33.33%, P > 0.05).

4. Discussion

Although many types of incubators have been successfully applied for human IVF, there were few studies compared the effects of the incubators with different

features on embryo development and clinical outcomes. We noticed two related reports about the effects of intra-incubator environment on embryo development; however, their results were inconsistent [5, 6]. Besides, it is worth noting that previous conclusions were based on 'one patient, one incubator' and strictly paired comparisons ('one patient, two types of incubators') were lacking. We allocated COCs from one patient equally and non-selectively to either incubator from fertilization to day-6-embryo-culture and found that the microenvironment of air jacket incubator could recover quickly, but there were no significant differences for embryo development and clinical outcomes between two types of incubators.

The pH levels, temperature, osmolality and humidity of embryo culture micro-environment are maintained by the incubator [6]. However, different incubators have different methods to heat, control gas and humidity. In our study, we found that the air jacket incubator represented a better performance in temperature recovering (including the atmosphere temperature and culture medium temperature) and O2 recovering. For air jacket incubators, the gas pre-processing tank was used to heat and mix different gas, after which the warmed and mixed gas (37°C, 6% CO2, 5% O2, and 89% N2) was released into the culture chamber. Besides, with a smaller volume and heated lid, the parameters of incubator micro-environment are easy to recover. On the contrary, for the conventional water jacked incubator, due to the large volume, the distance of thermal transmission to dishes is relatively long, which leads to a long time needed for temperature recovering. On the other hand, the gas control system is different between two types of incubators (**Figure 1C**). On–off control is a simple form of gas feedback control in conventional water jacket incubators, which drives CO2/O2 from fully closed to full open depending on the set point. Therefore, during the process of steady state recovering, the related parameters could fluctuate around the set point. However, the duty control is a stricter and precise method for gas control in air jacket incubators.

Although air jacket incubators have better properties than water jacket incubators, there were no significant differences in fertilization, embryo development and clinical outcomes observed in our study (**Tables 2** and **3**). As shown in **Figure 2B**, after 3 min operation outside of the incubator (still placed on a 37°C thermostatic platform), the temperature of culture medium dropped to 35°C. It appears that this low temperature in a short time might not affect embryo development. Actually, the temperatures in the cervix, oviduct and the ovary are between 36°C and 37°C [10]. Therefore, although a temperature recovering in water jacket incubators is longer, the short duration of limited low temperature did not affect embryo development and clinical outcomes. It was reported that incubator door openings could lead to measurable, significant changes in mouse embryo morphokinetics [11], suggesting that frequent disruption of intra-incubator environment is harmful for embryo development.

Overall, we concluded that, types of incubators could not affect embryo developmental competence and clinical outcomes as long as the intra-incubator environments are maintained to be stable and it should avoid frequent and prolonged door openings.

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Conflict of interest

The authors declare no conflict of interest.

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