International Journal of Animal Science and Technology
2023; 7(3): 43-47
http://www.sciencepublishinggroup.com/j/ijast
doi: 10.11648/j.ijast.20230703.12
ISSN: 2640-1282 (Print); ISSN: 2640-1312 (Online)

Effect of Stainless Steel Tank Incubation System on Development of Ovine Embryos

Feng Guangyu[1], Xiang Chunhe[1], Wang Limin[2, 3], Zhou Ping[2, 3], Pi Wenhui[1, *]

[1]College of Animal Science and Technology, Shihezi University, Shihezi, China

[2]State Key Laboratory of Sheep Genetic Improvement and Healthy Breeding, Xinjiang Academy of Agricultural and Reclamation Sciences, Shihezi, China

[3]Institute of Animal Husbandry and Veterinary Medicine, Xinjiang Academy of Agricultural and Reclamation Sciences, Shihezi, China

Email address:

846640756@qq.com (Feng Guangyu), 1512135977@qq.com (Xiang Chunhe), wanglm1980@126.com (Wang Limin), zhpxqf@163.com (Zhou Ping), wzjpwh@163.com (Pi Wenhui)

[*]Corresponding author

To cite this article:

Feng Guangyu, Xiang Chunhe, Wang Limin, Zhou Ping, Pi Wenhui. Effect of Stainless Steel Tank Incubation System on Development of Ovine Embryos. *International Journal of Animal Science and Technology*. Vol. 7, No. 3, 2023, pp. 43-47. doi: 10.11648/j.ijast.20230703.12

Received: September 15, 2023; **Accepted**: October 4, 2023; **Published**: October 14, 2023

Abstract: The utilization of separate culture chambers serves a dual purpose: it not only facilitates the creation of a hypoxic environment but also contributes to the establishment of a consistently stable local environment. This, in turn, mitigates environmental stress factors. The primary objective of this experiment was to assess the developmental potential of sheep embryos before their implantation. This assessment was conducted within the confines of 304 stainless steel culture chambers, offering a viable alternative for maintaining a stable in vitro embryo culture environment. The experiment involved a comparative analysis between the development outcomes of parthenogenetic and reconstituted sheep embryos cultured in both stainless steel tanks or MIC-101 incubators over a period of 7 days. Notably, the blastocyst rates for parthenogenetic embryos were 26.45% and 24.84% in stainless steel tanks and MIC-101 incubators, respectively, with no statistically significant difference (P>0.05) between them. Similarly, the blastocyst rates for reconstituted embryos stood at 16.07% and 16.84% (P>0.05) for stainless steel tanks and MIC-101 incubators, respectively. It is worth highlighting that the incubation system, comprising a 304 stainless steel tank, a standard gas mixture, and a thermostat, presents an in vitro culture system with the advantages of cost-effectiveness, environmental stability, and efficacy.

Keywords: Incubator, In Vitro Culture, Embryo, Sheep

1. Introduction

Mammalian oocytes and early embryos are highly sensitive to environmental stress. In the embryo culture laboratories, the carbon dioxide (CO_2) incubator emerges as an indispensable apparatus, serving as the principal habitat for gametes and embryos during in vitro cultivation. The main function of the CO_2 incubator is to establish and maintain a stable environment for the optimal development of gametes and embryos. To achieve this objective, the CO_2 incubator accurately adjusts parameters, encompassing temperature, CO_2 concentration, pH, oxygen levels, humidity/evaporation, and medium osmolarity, each of which exerts a profound influence on the trajectory of embryo development [1]. However, it is noteworthy that many commercially available CO_2 incubators possess expansive internal dimensions, and their day-to-day operation necessitates the frequent opening and closing of the incubator doors. This recurrent disruption of the incubator's internal equilibrium poses a potential challenge to the overall stability of the culture system.

In order to maintain environmental stability within individual units, most laboratories utilize dedicated incubators for personal use, reducing shared space and minimizing the frequency of incubator door openings. While this approach helps in stabilizing the environment, it may result in decreased equipment efficiency and an increase in fixed asset investments.

A submerged incubation system was reported by Vajta. In this method, culture dishes containing embryos are

individually enclosed within impermeable laminated foil bags, devoid of permeable carbon dioxide, oxygen, and nitrogen. These bags are filled with the required gas mixture, heat-sealed, and immersed in a circulating temperature-controlled water bath to achieve the desired culture period for up to 7 days. Consequently, all packaged culture dishes can function as individual incubators, often referred to as "submarines." The merits of this system encompass stable temperature, humidity, and gas mixture regulation. Additionally, these parameters can be rapidly restored upon opening. This approach allows for flexible utilization of various gas mixtures, ensuring safety, cost-effectiveness, reduced contamination risks, minimal cleanliness concerns, and convenient transport [2, 3].

In this study, culture dishes containing early-stage embryos were individually placed within 1-liter 304 stainless steel pressure vessels. These vessels were then filled with a gas mixture at saturated humidity levels. After sealing the vessels, they were placed inside a constant-temperature incubator for 7 days of cultivation. The incubation system maintains a stable environment within each culture unit. Each pressure vessel can function as an independent culture unit, offering the advantages exhibited by the submerged incubation system.

2. Materials and Methods

2.1. Ethical Approval

The experiment was carried out in compliance with all rules and regulations regarding animal rights established by the Bioethics Committee of Shihezi University.

2.2. Main Reagents

TCM199 medium for in vitro maturation of oocytes was purchased from Gibco Company (Cat#11150-059). The following solutions were prepared in-house: in vitro handling solution (H199), early embryo in vitro culture and development solution (SOFaai: NaCl 107.70mM, KCl 7.15mM, $NaHCO_3$ 25.00mM, KH_2PO_4 0.30mM, Na Lactate 3.32mM, pyruvate 0.33mM, $CaCl_2*2H_2O$ 1.71mM, Myo-inositol 2.77mM, GlutaMAXTM 1mM, MEM non-essential amino acids 100×, Basal medium Eagles essential amino acids 50×, Gentamicin 50mg/L, fatty acid-free BSA 8mg/ml) [4], electrofusion solution, and activation solution (SOFaai+7% ethanol and SOFaai+2μmol/L 6-DMAP), as previously described [5].

2.3. Experimental Materials

The 1L 304 stainless steel pressure vessel was purchased from Suzhou Ruixiang Electronics Store. The MIC-101 cultivation system (Billups-Rothenberg) had a volume of approximately 8L per cultivation chamber. A Heraeus carbon dioxide incubator cannot function properly due to damage to the carbon dioxide concentration detector. This incubator on the market had been discontinued and could not match the corresponding carbon dioxide detector. Due to the normal temperature control system, it was used as a constant temperature incubator in our laboratory. Standard premixed gas (5% CO_2, 7% O_2, and 88% N_2) was purchased from Shanghai Weichuang Standard Gas Co., Ltd. Ovaries of sheep were sourced from a local slaughterhouse.

2.4. Experimental Methods

2.4.1. Starvation Cultivation of Primary Ovine Fibroblast Cells

Skin tissue was taken from the ears of a 3-year-old Suffolk ram using sterile procedures, washed and cut into pieces, and cultured in DMEM+10% FBS with dual antibody solution. In the primary culture, the medium was changed every 3 days. After 5 days, fibroblast cells grew to approximately 90% confluence. The cells were then dissociated using trypsin, centrifuged, and washed. Subsequently, they were passaged in DMEM supplemented with 10% FBS, with the medium being changed every 3 days. Three generations of cells were passaged and frozen for storage. After thawing, the cells were inoculated on a 24 well plate for culture. When the cell growth reached 80% confluence, washed twice with PBS and cultured in DMEM medium with 2% FBS, changing the medium every 3 days. Cells were cultured using serum starvation method until the 6th day, digested with trypsin, centrifuged at 2500 r/mim and collected into 1.5 mL EP tubes. The cells were resuspended in 2% FBS DMEM solution as nuclear donors.

2.4.2. Sheep Somatic Cell Nuclear Transfer

The ovaries were immersed in a thermos bottle of physiological saline at approximately 30°C and transported to the laboratory within 120 minutes for processing. The ovaries were washed twice in PBS and then placed in a culture dish containing Hepes-buffered TCM-199 with 60 IU/mL heparin. Using a surgical blade, the follicles on the surface of the ovaries were dissected. Dense cumulus-oocyte complexes (COCs) were selected and washed twice in Hepes-buffered TCM-199. Groups of 50 COCs were matured in 400 μl of $NaHCO_3$-buffered TCM-199, supplemented with 10% (V:V) fetal bovine serum (FBS), 2 mM GlutaMAXTM, 0.3 mM sodium pyruvate, 0.1 mM cysteine, 5 μg/mL follicle-stimulating hormone, 5 μg/mL luteinizing hormone, and 1 μg/mL β-estradiol. They were then placed in a culture chamber maintained at 38.6°C with 5% CO_2 saturation and high humidity.

COCs were matured for 22 hours, and cumulus cells were removed in Hepes-buffered TCM-199 containing 1 mg/mL hyaluronidase. All procedures were carried out on a 36°C heated plate. High-quality mature oocytes extruding with the first polar body were selected for further use.

In a 60 mm culture dish, an operation droplet was prepared (M199+25 mmol/L Hepes+20% FBS+7.5μg/ml Cytochalasin) and covered with mineral oil. Nuclear removal and injection were performed on a micromanipulation stage. Reconstructed oocyte-nucleus complexes were cultured in the maturation medium for 30 minutes. Then, electrofusion was performed in fusion medium (0.3 mol/L mannitol+0.5 mmol/L Hepes+0.1 mmol/L $CaCl_2$+0.1 mmol/L $MgCl_2$) under the conditions of a direct current pulse field strength of 25 kV/cm, duration of 20

μs, and two pulses. Electrofusion was performed in batches of 5–8 complexes at a time.

After electrofusion, the oocyte/nucleus complexes were cultured for 45 minutes to observe fusion. Unfused complexes were removed. Reconstructed embryos were further cultured for 2 hours for activation treatment, which involved 7% ethanol for 7 minutes and 2 μmol/L 6-DMAP for 4 hours. After washing the reconstructed embryos, they were transferred to pre-equilibrated development medium SOFaai.

Culture dishes containing reconstructed embryos were placed in a culture chamber (1L 304 stainless steel pressure vessel or MIC-101 cultivation system). The chamber was filled with a mixed gas of distilled water (5% CO_2, 7% O_2, and 88% N_2). The chamber was sealed, and it was placed in a constant temperature incubator at 38.6°C. On the 7th day, the chamber was opened, and the blastocyst rate was examined.

2.4.3. Production of Parthenogenetic Embryos

After 24 h of in vitro maturation, granule cells around the oocytes were digested and removed. The oocytes were subjected to activation treatment with 7% ethanol for 7 minutes and 2 μmol/L 6-DMAP for 4 hours. They were then washed three times with SOFaai. The parthenogenetic embryos were placed in pre-equilibrated development medium SOFaai and cultured in sealed culture chambers (1L 304 stainless steel pressure vessel or MIC-101 cultivation system) at 38.6°C, 5% CO_2, 7% O_2, and 88% N_2 saturation with high humidity. On the 7th day of culture, the blastocyst rate was examined.

3. Statistical Analysis

The experimental data were analyzed using a chi-square (X^2) test, with $P<0.05$ considered statistically significant.

4. Results and Discussion

4.1. In Vitro Development Results of Parthenogenetic Embryos

Parhenogenetic embryos were cultured in different culture vessels, and the effect of different culture systems on the development of parthenogenetic embryos was determined by the blastocyst rate on the 7th day. The results of 308 parthenogenetic embryos in 3 replicates showed no difference in blastocyst development rate in 304 stainless steel tanks and MIC-101 culture system (26.45% vs 24.84%, $P>0.05$).

Table 1. *Effects of Different Incubators on the In Vitro Development of Parthenogenetic Embryos.*

Incubator	Number of Repetitions	Number of Parthenogenetic Oocytes	Blastocyst Rate on the 7th Day
MIC-101 Cultivation System	3	153	24.84% (38)
304 Stainless Steel Tank	3	155	26.45% (41)

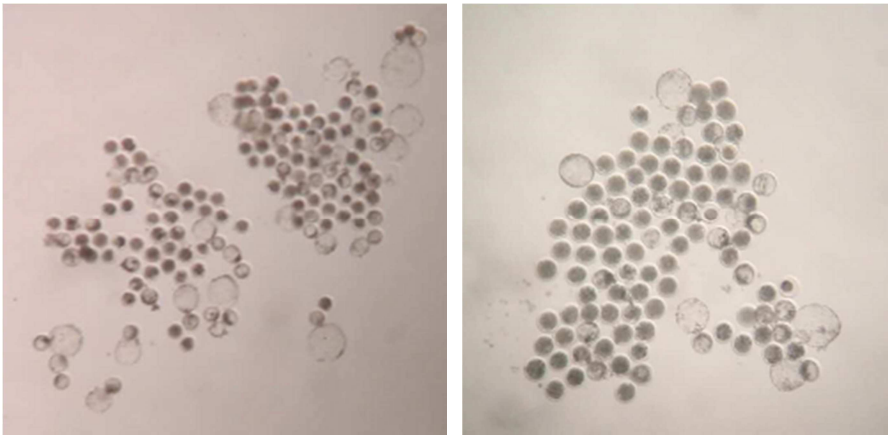
4.2. In Vitro Development Results of Cloned Reconstructed Embryos

The reconstructed embryos were cultured in different culture vessels. The impact of different culture vessels on the in vitro development of reconstructed embryos was determined according to the blastocyst rate on the 7th day. The experiment involved four repetitions and a total of 948 reconstructed embryos.

The results showed that there was no significant difference in the blastocyst development rate of cloned reconstructed embryos between the 304 stainless steel pressure vessel and the MIC-101 cultivation system (16.07% vs. 16.84%, $P>0.05$).

Table 2. *Effects of Different Incubators on the In Vitro Development of Reconstructed Embryos.*

Incubator	Number of Repetitions	Number of Reconstructed Embryos	Blastocyst Rate on the 7th Day
MIC-101 Cultivation System	4	475	16.84% (80)
304 Stainless Steel Tank	4	473	16.07% (76)



MIC-101 Cultivation System 304 Stainless Steel Tank

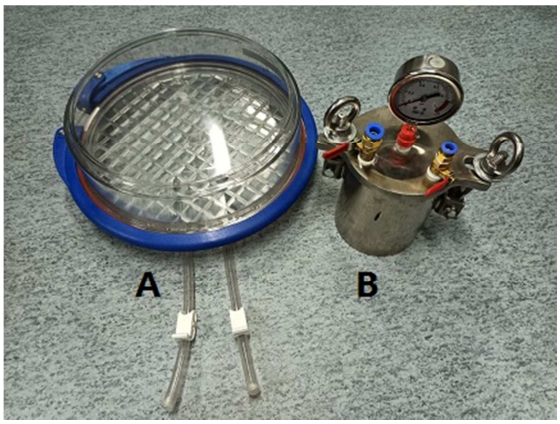
Figure 1. *Development of Reconstructed Embryos in Different Incubators.*

Under an inverted microscope, the morphology of blastocysts, inner cell mass, and hatching outcomes were observed, and no differences were observed between the two culture systems (see Figure 1), suggesting that both MIC-101 and the 304 stainless steel tank incubation systems meet the requirements for early embryo in vitro culture. Figure 1 is a photograph taken with a mobile phone through the eyepiece of the inverted microscope.

4.3. Discussion

The oxygen concentration in the oviduct and uterus of almost all investigated species is significantly lower than that in the atmosphere (1.5-6% compared to 21%) [6]. For early embryo in vitro culture, cattle [7], pigs [8], sheep [9], mouse [10] and human [11] have been shown to have better developmental outcomes and embryo quality when using simple culture media (non-co-culture systems) and culturing in a low-oxygen (5%-8%) environment compared to atmospheric conditions. This has been linked to improved embryo pregnancy rates. Low oxygen concentration should be a principle for all mammalian embryo culture systems, including humans [12], and there is a global consensus on this matter [6].

The MIC-101 cultivation system (seen in Figure 2A) has been adopted by many laboratories for cell, embryo, or other biological culture purposes. It provides a controlled atmospheric environment with constant gas concentrations, stable humidity, and isolation from potential contaminants. The MIC-101 system allows for precise experimental conditions to be maintained and can be adjusted as needed for variations in gas concentration, humidity, or temperature, making it user-friendly.



A: MIC Cultivation System; B: 304 Stainless Steel Tank Cultivation System

Figure 2. *Photographs of the Two Incubators.*

The 304 stainless steel tank (seen in Figure 2B) shares the same stainless steel inner lining as most carbon dioxide incubators, making it suitable for embryo culture. The 1L 304 stainless steel tank used in the experiment has a relatively low cost, around 300 RMB per unit, making it an economical choice. With a chamber volume of 1L, it is smaller than the MIC-101 system (approximately 8L), saving on the consumption of mixed gas and taking up less space in the incubator, thus increasing laboratory equipment efficiency. In a closed system, 50ml of mixed gas can meet the needs of 200 cattle embryos from fertilization to the blastocyst stage at 7 days [2]. Therefore, if only embryo culture is required, a smaller stainless steel tank can be used.

Compared to MIC-101 or laminated foil bags, stainless steel tanks can withstand certain levels of pressure. Pressure can induce stress in embryos, and appropriate stress may improve embryo quality [13]. Therefore, using a 304 stainless steel tank as an incubator for early embryo culture can provide a foundational apparatus for studying the effects of pressure on in vitro embryo development.

5. Conclusion

Creating an optimal environment for gametes and embryos constitutes a fundamental principle adhered to by all embryo engineering laboratories [14]. The reduced oxygen environment enhances the metabolic activity of embryos, particularly those cultured under lower glucose concentrations, resulting in accelerated kinetics [15]. Independent culture chambers can not only achieve a low-oxygen environment, but also help to achieve a more stable local environment and reduce environmental stress. The experimental results showed that there was no significant difference ($P > 0.05$) between the blastocyst development rate of parthenogenetic embryos (26.45% vs. 24.84%) and the blastocyst development rate of reconstructed embryos (16.07% vs. 16.84%) between the stainless steel tank system and the MIC-101 culture system at 5% CO_2, 7% O_2, 88% N_2 standard mixture, saturated humidity and 38.6°C.

Conflict of Interests

All the authors do not have any possible conflicts of interest.

Acknowledgments

Startup Fund for Scientific Research of High-level Talents of Shihezi University (RCZK2021B34); Supported by the scientific research project of Xinjiang Production and Construction Corps.

References

[1] Swain JE. Decisions for the IVF laboratory: comparative analysis of embryo culture incubators. Reproductive biomedicine online vol. 28 (5): 535-547, 2014. DOI: 10.1016/j.rbmo.2014.01.004.

[2] Vajta G, Holm P, Greve T, Callesen H. The submarine incubation system, a new tool for in vitro embryo culture. A technique report. Theriogenology. 48 (8): 1379–1385, 1997. DOI: 10.1016/S0093-691X(97)00379-8.

[3] Vajta G, Bartels P, Joubert J, de la Rey M, Treadwell R, Callesen H. Production of a healthy calf by somatic cell nuclear transfer without micromanipulators and carbon dioxide incubators using the Handmade Cloning (HMC) and the Submarine Incubation System (SIS). Theriogenology. 62 (8): 1465-1472, 2004. DIO: 10.1016/j.theriogenology.2004.02.010.

[4] Speckhart SL, Wooldridge LK, Ealy AD. An updated protocol for in vitro bovine embryo production. STAR Protoc. 4 (1): 101924, 2022. DOI: 10.1016/j.xpro.2022.101924.

[5] Guo YH, Zhang YY, Wang LM, Tang H, Li YL, Zhou Ping. Program optimization before enucleation on ovine somatic cell nuclear transfer. Chinese Journal of Biotechnology. 33 (5): 730-742, 2017. DOI: 10.13345/j.cjb.160308.

[6] Vajta G, Rienzi L, Cobo A, Yovich J. Embryo culture: can we perform better than nature. Reproductive BioMedicine Online. 20 (4): 453-469, 2010. DOI: 10.1016/j.rbmo.2009.12.018.

[7] Nagao Y, Saeki K, Hoshi M, Kainuma H. Effects of oxygen concentration and oviductal epithelial tissue on the development of in vitro matured and fertilized bovine oocytes cultured in protein-free medium. Theriogenology. 41 (3): 681–687, 1994. DOI: 10.1016/0093-691x(94)90177-k.

[8] Karja NWK, Wongsrikeao P, Murakami M, Agung B, Fahrudin M, Nagai T, Otoi T. Effects of oxygen tension on the development and quality of porcine in vitro fertilized embryos. Theriogenology 62 (9): 1585-1595, 2004. DOI: 10.1016/j.theriogenology.2004.03.012.

[9] Leoni GG, Rosati I, Succu S, Bogliolo L, Bebbere D, Berlinguer F, Ledda S, Naitana S. A Low Oxygen Atmosphere during IVF Accelerates the Kinetic of Formation of In Vitro Produced Ovine Blastocyst. Reproduction in Domestic Animals. 42 (3): 299-304, 2007. DOI: 10.1111/j.1439-0531.2006.00783.x.

[10] Lee SC, Seo HC, Lee J, Jun JH, Choi KW. Effects of dynamic oxygen concentrations on the development of mouse pre- and peri-implantation embryos using a double-channel gas supply incubator system. Clin Exp Reprod Med. 46 (4): 189-196, 2019. DOI: 10.5653/cerm.2019.00514.

[11] Li M, Xue X, Shi J. Ultralow Oxygen Tension (2%) Is Beneficial for Blastocyst Formation of In Vitro Human Low-Quality Embryo Culture. Biomed Res Int. 9603185, 2022. DOI: 10.1155/2022/9603185.

[12] Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, Barnett BD, Madden JD. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. Human Reproduction. 24 (2): 300–307, 2009. DOI: 10.1093/humrep/den368.

[13] Du Y, Lin L, Schmidt M, Bøgh IB, Kragh PM, Sørensen CB, Li J, Purup S, Pribenszky C, Molnár M, Kuwayama M, Zhang X, Yang H, Bolund L, Vajta G. High hydrostatic pressure treatment of porcine oocytes before handmade cloning improves developmental competence and cryosurvival. Cloning Stem Cells. 10 (3): 325–330, 2008. DOI: 10.1089/clo.2007.0089.

[14] Knudtson JF, Robinson RD, Sparks AE, Hill MJ, Chang TA, Van Voorhis BJ. Common practices among consistently high-performing in vitro fertilization programs in the United States: 10-year update. Fertil Steril. 117 (1): 42-50, 2022. DOI: 10.1016/j.fertnstert.2021.09.010.

[15] de Lima CB, Dos Santos ÉC, Ispada J, Fontes PK, Nogueira MFG, Dos Santos CMD, Milazzotto MP. The dynamics between in vitro culture and metabolism: embryonic adaptation to environmental changes. Sci Rep. 10 (1): 15672, 2020. DOI: 10.1038/s41598-020-72221-1.