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Effects of changing culture medium on preimplantation embryo development in rabbit

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Research Article

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Summary

Many studies have focused on the optimization of the composition of embryo culture medium; however, there are few studies involving the effect of a culture medium changing procedure on the preimplantation development of embryos. In this study, three groups were designed: a non-renewal group, a renewal group and a half-renewal group. The levels of reactive oxygen species (ROS), apoptotic index, blastocyst ratio and blastocyst total cell number were analyzed in each group. The results showed that the ROS level and the apoptotic index of blastocyst in the non-renewal group were significantly higher than in the renewal group and the half-renewal group ($P < 0.05$). The blastocyst ratio and blastocyst total cell number were significantly higher in the half-renewal group than that in non-renewal group and the renewal group ($P < 0.05$). These results demonstrated that the procedure of changing the culture medium influenced ROS level, apoptotic index, blastocyst ratio and total cell number of blastocysts. In addition, the result suggested that changing the culture medium may lead to a loss of important regulatory factors for embryos, while not changing the culture medium may lead to the accumulation of toxic substances. Half-renewal can alleviate the defects of both no renewal and renewal, and benefit embryo development. This study will be of high value as a reference for the optimization of embryo culture *in vitro*, and is very significant for assisted reproduction.

Introduction

Infertility is increasingly acknowledged as a global public health issue by the World Health Organization, and a growing number of couples currently worry or have previously worried about infertility (De Neubourg *et al.*, 2012; Barratt *et al.*, 2017). Since the first *in vitro* fertilization (IVF) baby was born in 1978, more than 5 million IVF babies have been born worldwide, and the number is increasing exponentially (Faddy *et al.*, 2018). Embryo culture is a crucial step for IVF and other assisted reproduction techniques, and the embryo culture medium has a wide influence on embryo development in terms of metabolism, morphology, epigenetic conditions, gene expression and developmental ability (Mani and Mainigi, 2018). It has been reported that IVF babies have a higher risk of cancer, metabolic diseases, hypertension, and cardiovascular diseases, and this may be closely related to *in vitro* culture (Simonstein *et al.*, 2014).

Many kinds of embryo culture medium are at this time available for humans and animals (Rossi *et al.*, 2019). Most research has focused on optimizing the composition of the culture medium, and only a few studies have tackled the culture medium changing procedure (de los Santos *et al.*, 2015). Studies have shown that *in vitro* cultured embryos can secrete some important factors into the medium that play an important role in regulating their development (Kim *et al.*, 2016; Lin *et al.*, 2019). At the same time, embryo metabolites or degradation products of the culture medium will produce some substances that are harmful to the embryo cultured *in vitro* (Shohael *et al.*, 2006). Renewal of the embryo culture medium may lead to the loss of some important secretions, while non-renewal of the embryo culture medium may lead to the accumulation of toxic substances.

Rabbits play an important role in reproductive biology (Fischer *et al.*, 2012). Many pioneering achievements in reproductive biology come from research using rabbits (Alexandre 2001; Fischer *et al.*, 2012). In this study, the rabbit was used as a model to study the effect of changing the culture medium on embryo development *in vitro*, and our work will provide an important reference for the optimization of embryo culture.

Materials and methods

Animal

Here, 6–8-month-old rabbits were raised individually in cages at the Laboratory Animal Center of Xi'an Jiaotong University with unrestricted access to food and water. The temperature was

maintained at *c.* 25°C, and the day:night ratio was 12 h:12 h. All procedures involved in the animal experiments were in line with animal ethics and welfare requirements to minimize animal suffering.

Collection of fertilized eggs

Here, 80 IU pregnant mare serum gonadotropin (PMSG; Shusheng, Ningbo, China) was injected subcutaneously, then hCG (SHUSHENG, Ningbo, China) 100 IU was injected intravenously 72 h later, and the female rabbits were immediately mated with male rabbits. At 20–22 h after mating, the female rabbits were anaesthetised, and the abdominal cavity was opened to separate the fallopian tube. A syringe loaded with 2 ml of Dulbecco's phosphate-buffered saline (DPBS) containing 5% fetal bovine serum was inserted into the fallopian tube near the uterine segment, and the end of the fallopian tube near the ovary was placed in a centrifuge tube. Then the plunger of the syringe was pressed down to flush out the fertilized eggs. The fertilized eggs were collected under a stereomicroscope and transferred into DPBS containing hyaluronidase (5 mg/l).

In vitro culture

The fertilized embryos were treated with DPBS containing hyaluronidase for 5 min at 38.5°C, and then blown gently to remove the granulosa cells from the embryos. The embryos were then transferred into embryo culture medium covered with oil. The formula of the embryo culture medium is shown in Table 1. The amount of culture medium in each culture dish was 400 µl, the number of embryos in each dish was 40, and the culture environment was maintained at 38.5°C, at saturated humidity and with 5% CO_2. Three groups were designated, a non-renewal group, a renewal group, and a half-renewal group. In the non-renewal group, embryo culture medium was not changed from day 1 to day 4. In the renewal group, embryo culture medium was completely changed on day 3. In the half-renewal group, 200 µl of old culture medium was drawn out on day 3, and replaced with 200 µl fresh culture medium.

ROS staining

Embryos were incubated for 20 min in serum-free medium containing 10 mM 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) at 37°C. Then the embryos were washed three times in serum-free medium, and examined and photographed using a Nikon Eclipse TI-s microscope (Nikon, Tokyo, Japan), and analyzed using Image Pro Plus software (Media Cybernetics, Bethesda, MD).

Apoptosis staining

A terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) kit (Promega, Madison, WI, USA) was used to analyze the apoptosis of blastocysts. Embryos were washed three times with 0.2% PBS-polyvinyl alcohol (PBS-PVA) for 5 min each time, and then fixed with 4% paraformaldehyde at room temperature (RT) for 30 min. Embryos were washed three times with 0.2% PBS-PVA for 5 min each time. The washed embryos were then equilibrated in E-buffer for 5–8 min, and then transferred to apoptotic dye solution containing 45 µl E-buffer, 5 µl nucleotide mix and 1 µl rTDT at 37°C for 1 h. The reaction was terminated by applying saline–sodium citrate (SSC) buffer for 15 min, and the embryos were washed three times with 0.2% PBS-PVA. Embryos were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 3 min, then the embryos were washed three times with 0.2% PBS-PVA. Next the embryos were transferred to a glass slide, pressed and covered with glass, and sealed with nail polish. Samples were examined and photographed under a Nikon Eclipse TI-s microscope (Nikon), and analyzed using Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

Table 1. Composition of embryo culture medium

Component	Units	Content
NaCl	mM	110
KCl	mM	7.168
KH_2PO_4	mM	1.191
$CaCl_2.2H_2O$	mM	1.707
$MgCl_2.6H_2O$	mM	0.4926
$NaHCO_3$	mM	25.07
Na-pyruvate	mM	0.3
Glucose	mM	1.498
Na-lactate	%	0.02824
EAA (50×)	%	2
NEAA (100×)	%	1
BSA	mg/ml	8

Counting total number of cells in blastocysts

Blastocysts were treated with DPBS containing hyaluronidase to remove the granulosa cells. Then the blastocysts were washed three times with 0.2% PBS-PVA for 5 min each time, and fixed with 4% paraformaldehyde at RT for 30 min. The blastocysts were treated with DAPI for 3 min, and then were washed three times with 0.2% PBS-PVA. After transfer onto a glass slide and being pressed with a coverslip, the blastocysts were examined and photographed under a Nikon Eclipse TI-s microscope (Nikon), and counted using Image Pro Plus software (Media Cybernetics).

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Blastocyst rate was analyzed using the chi-squared test. The relative level of ROS, apoptotic index of blastocysts, and blastocyst total cell number were analyzed using one-way analysis of variance (ANOVA). Each experiment was repeated more than three times.

Results

Effect of changing the culture medium on the ROS level of blastocysts

Embryos in the non-renewal, renewal and half-renewal groups were cultured for 4 days, and the relative level of ROS of the blastocysts was analyzed using DCHF-DA. The result showed that the ROS level of blastocysts in the non-renewal group was significantly higher than in the renewal group and the half-renewal group

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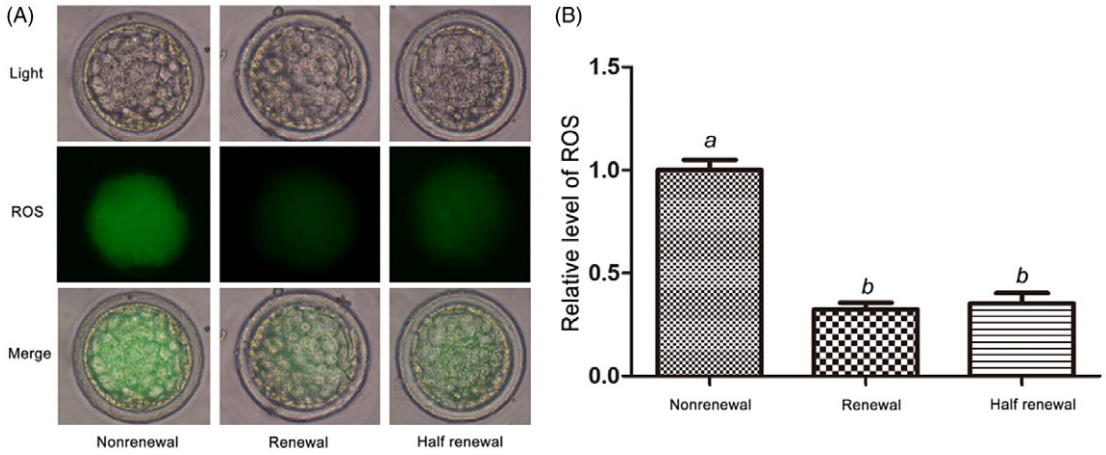

Figure 1. Relative level of ROS in blastocysts in the non-renewal, renewal and half-renewal groups. (A) ROS staining in blastocysts. Upper row: bright field; middle row: green fluorescence indicating ROS; lower row: merged bright field and green fluorescence. (B) Quantification of ROS fluorescence intensity. Different letters above the bars indicate significant differences ($P < 0.05$).

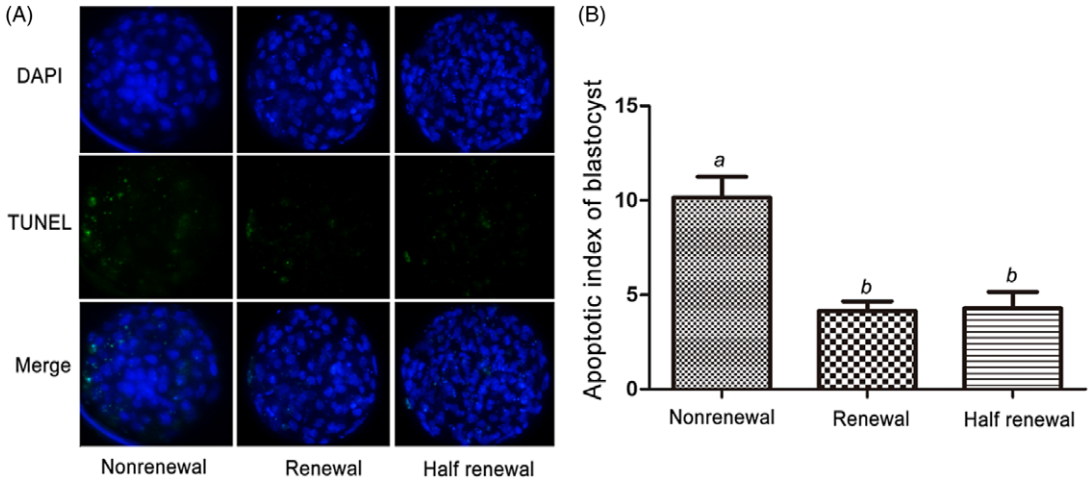

Figure 2. Apoptosis index of blastocysts in the non-renewal, renewal and half-renewal groups. (A) TUNEL assay of apoptotic blastomeres. Upper row: DAPI; middle row: green fluorescence indicating apoptotic cells; bottom row: merged DAPI and apoptotic cell. (B) Apoptosis index of blastocysts. Different letters above the bars indicate significant differences ($P < 0.05$).

($P < 0.05$), and that there was no significant difference between the renewal group and the half-renewal group ($P > 0.05$; Fig. 1).

Effect of changing the culture medium on the apoptotic index of blastocysts

On day 4 blastocysts were collected and analyzed using a TUNEL kit in each group. The apoptotic index was defined as the number of apoptotic cells divided by the blastocyst total cell number. The result showed that the apoptotic index of blastocysts in the non-renewal group was significantly higher than in the renewal group and the half-renewal group ($P < 0.05$), and that the difference between the renewal group and the half-renewal group was not significant ($P > 0.05$; Fig. 2).

Effect of changing the culture medium on the blastocyst ratio and blastocyst total cell number

The blastocyst ratio and the blastocyst total cell number in each group were analyzed on day 4. The results showed that the blastocyst ratio (Fig. 3A) and blastocyst total cell number (Fig. 3B) were significantly higher in the half-renewal group than in the non-renewal group and the renewal group. The blastocyst ratio and the blastocyst total cell number in the non-renewal group and the renewal group showed no significant difference ($P < 0.05$).

Discussion

Much progress has been made in the optimization of embryo culture medium, and two different approaches exist to embryo culture: one attitude is 'let the embryo choose', the other is 'return to nature' (Koscinski *et al.*, 2018). Guided by the first tactic, culture medium' formula was determined by experience, experiment and algorithm analysis, allowing the culture medium to support all nutrient requirements from the zygote to the blastocyst stage (Thouas *et al.*, 2003). For example, KSOM medium was developed based on this idea and has been successfully used in mice, rabbits and cattle (Liu *et al.*, 1995; Jin *et al.*, 2000; Zhu *et al.*, 2004; Men

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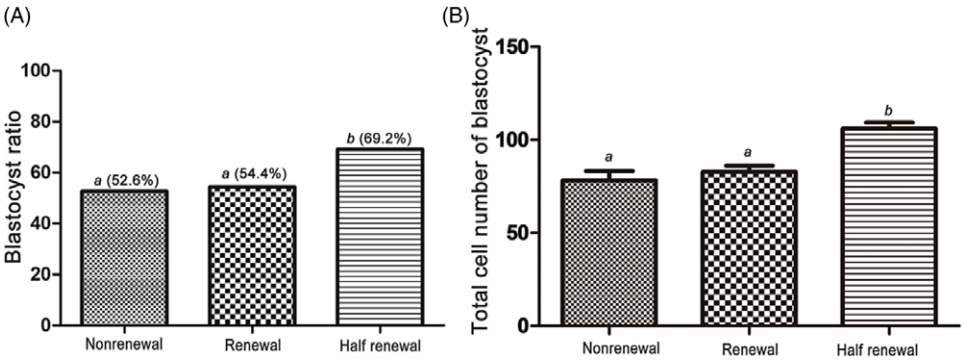

Figure 3. Blastocyst ratio and blastocyst total cell number in non-renewal, renewal and half-renewal groups. (A) Blastocyst ratio on day 4 in non-renewal, renewal and half-renewal groups. (B) Total cell number of blastocysts in non-renewal, renewal and half-renewal groups. Different letters above the bars indicate significant differences ($P < 0.05$).

et al., 2020). Under the guidance of 'return to nature', culture medium was synthesized by defining the different nutrients required at different stages of embryonic development, simulating the components of oviduct fluid or uterine fluid *in vivo* (Qu *et al.*, 2020a). In the zygote and the first few cleavage stages, the metabolic activity of the embryo is relatively weak (Pantaleon *et al.*, 2001). With the activation of the embryo's genome, the embryo migrates from the fallopian tube to the uterus, and during this process, the metabolism of the embryo gradually increases (Yang *et al.*, 2020). G1/G2 culture media were designed based on this. G1 culture medium was used at the first few cleavages, and then embryos were transferred into G2 culture medium according to the changes of embryonic development and nutritional requirements (Hall-Woods *et al.*, 2000; Sanches *et al.*, 2013). For KSOM medium, there was no need to renew the culture medium, but for G1/G2 culture medium, renewal of the culture medium was necessary.

During its development, the embryo is in close contact with the microenvironment of the embryo culture medium or oviduct fluid (Sekirina and Neganova, 1995; Lee *et al.*, 2017; Chen and Schoen, 2019). Renewal of the culture medium may lead to the loss of these important factors, which is not conducive to embryonic development. conversely, with increasing duration of the culture, some nutrients will be consumed, and toxic substances will accumulate as a result of decomposition or metabolism (Kleijkers *et al.*, 2016). In this study, three groups were designed: a non-renewal group, a renewal group and a half-renewal group. The ROS level, apoptotic index, embryonic development-related genes, blastocyst ratio and blastocyst total cell number were analyzed to study the effect of changing the culture medium on preimplantation embryos.

Previous studies have reported that the ROS level of embryos *in vitro* is higher than that of embryos developed *in vivo* (Amoushahi *et al.*, 2018; An *et al.*, 2019; Qu *et al.*, 2020b). Excessive ROS levels could damage lipid, protein and DNA, disturb gene expression, and block embryo development (Takahashi, 2012; Alarifi *et al.*, 2014). At appropriate concentrations, ROS can be used as a signal molecule participating in the regulation of the cell cycle, cell differentiation and development (Yang *et al.*, 2017). Treating the embryo *in vitro* with antioxidants, such as melatonin and glutathione, can effectively reduce ROS level and improve the ability of embryos to develop *in vitro* (Huang *et al.*, 2018; Li *et al.*, 2018; Pang *et al.*, 2019; Hicks *et al.*, 2020). In this study, the ROS level of blastocysts was significantly higher in the non-renewal group than in the renewal group and the half-renewal group, and there was no significant difference between the renewal group and the half-renewal group. Previous studies have reported that the concentration of ammonia in the culture medium increased with culture time, which led to an increase of the embryonic ROS level (Kleijkers *et al.*, 2016). The results indicated that oxides may accumulate in the culture medium during embryo culture, and the oxides might be removed or diluted by renewing culture medium, which could decrease the ROS level of the embryo.

In the process of embryonic development, many factors can lead to apoptosis (Qu *et al.*, 2020a). The apoptotic ratio of blastocysts was significantly increased under endoplasmic reticulum stress or heat stress (Lin *et al.*, 2016). In a hyperoxic environment, embryos showed a higher apoptotic ratio than in a hypoxic environment (Lin *et al.*, 2016). Pesticides and other toxicants increased the apoptotic ratio and caused embryonic malformation (Jia *et al.*, 2019). There is a close relationship between the apoptotic ratio and embryonic development potential, and a higher apoptotic ratio is considered to have a lower developmental potential (Jia *et al.*, 2019). Adding antioxidants or anti-stress compounds to the culture medium can effectively reduce the apoptotic ratio and enhance the development of the embryo *in vitro* (Lin *et al.*, 2016). In this study, we found that the apoptotic ratio of blastocysts was significantly higher in the non-renewal group than in the renewal group and the half-renewal group, and that the difference between the renewal group and the half-renewal group was not significant, indicating that non-renewal of embryo medium may not be conducive to embryonic development, while changing the culture medium influenced the apoptosis of embryos *in vitro*.

The blastocyst ratio and blastocyst total cell number are important indexes to evaluate embryo development. Studies have shown that embryos with a higher blastocyst ratio and blastocyst total cell number have higher developmental potential (Jia *et al.*, 2019). In this study, the blastocyst ratio and the blastocyst total cell number in the half-renewal group were significantly higher than those in the renewal group and the non-renewal group. This further confirms our hypothesis that changing the culture medium may lead to the loss of important regulatory factors for embryos, while not changing the culture medium may lead to the accumulation of toxic substances, which are both not conducive to embryonic development. Half-renewal can alleviate the defects of non-renewal or renewal, and benefit embryo development.

In conclusion, we found that the culture medium changing procedure could affect the level of ROS, the apoptotic index of blastocysts, the development rate of blastocysts and the blastocyst total cell number. This study has a high value as reference for

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the optimization of embryo culture *in vitro*, and has important significance for assisted reproduction.

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Conflicts of interest. The authors have no conflicts of interest to report regarding the present study.

Ethical standards. The Animal Care and Use Committee of Xi'an Jiaotong University approved all animal use procedures applied in this study.

References

Alarifi S, Ali D, Verma A, Almajhdi FN and Al-Qahtani AA (2014). Single-walled carbon nanotubes induce cytotoxicity and DNA damage via reactive oxygen species in human hepatocarcinoma cells. *In Vitro Cell Dev Biol Anim* **50**, 714–722.

Alexandre H (2001). A history of mammalian embryological research. *Int J Dev Biol* **45**, 457–67.

Amoushahi M, Salehnia M and Ghorbanmehr N (2018). The mitochondrial DNA copy number, cytochrome c oxidase activity and reactive oxygen species level in metaphase II oocytes obtained from *in vitro* culture of cryopreserved ovarian tissue in comparison with *in vivo*-obtained oocyte. *J Obstet Gynaecol Res* **44**, 1937–46.

An QL, Peng W, Cheng YY, Lu ZZ, Zhou C, Zhang Y and Su JM (2019). Melatonin supplementation during *in vitro* maturation of oocyte enhances subsequent development of bovine cloned embryos. *J Cell Physiol* **234**, 17370–81.

Barratt CLR, Björndahl L, De Jonge CJ, Lamb DJ, Osorio Martini FO, McLachlan R, Oates RD, van der Poel S, St John B, Sigman M, Sokol R and Tournaye H (2017). The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Hum Reprod Update* **23**, 660–80.

Chen S and Schoen J (2019). Air–liquid interface cell culture: from airway epithelium to the female reproductive tract. *Reprod Domest Anim* **54** Supplement 3, 38–45.

De Neubourg D, van Duijnhoven NTL, Nelen WLDM and D'Hooghe TM (2012). Dutch translation of the ICMART-WHO revised glossary on ART terminology. *Gynecol Obstet Invest* **74**, 233–48.

Faddy MJ, Gosden MD and Gosden RG (2018). A demographic projection of the contribution of assisted reproductive technologies to world population growth. *Reprod Biomed Online* **36**, 455–8.

Fischer B, Chavatte-Palmer P, Viebahn C, Navarrete Santos AN and Duranthon V (2012). Rabbit as a reproductive model for human health. *Reproduction* **144**, 1–10.

Hall-Woods ML, Krisher RL, Lane M, Gardner DK and Asa CS (2000). Comparison of Gardner's G1/G2 sequential media and buffalo rat liver (BRL) cell go-culture for bovine in-vitro embryo production. *Biol Reprod* **62**, 316.

Hicks E, Mentler M, Arena HA, Current JZ and Whitaker BD (2020). Cyanidin improves oocyte maturation and the *in vitro* production of pig embryos. *In Vitro Cell Dev Biol Anim* **56**, 577–84.

Huang ZQ, Pang YW, Hao HS, Du WH, Zhao XM and Zhu HB (2018). Effects of epigallocatechin-3-gallate on bovine oocytes matured *in vitro*. *Asian Australas J Anim Sci* **31**, 1420–30.

Jia LY, Ding B, Shen C, Luo SW, Zhang YR, Zhou L, Ding RK, Qu PX and Liu EQ (2019). Use of oocytes selected by brilliant cresyl blue staining enhances rabbit cloned embryo development *in vitro*. *Zygote* **27**, 166–72.

Jin DI, Kim DK, Im KS and Choi WS (2000). Successful pregnancy after transfer of rabbit blastocysts grown *in vitro* from single-cell zygotes. *Theriogenology* **54**, 1109–16.

de Los Santos MJ, Gámiz P, de Los Santos JM, Romero JL, Prados N, Alonso C, Remohí J and Dominguez F (2015). The metabolomic profile of spent culture media from Day-3 human embryos cultured under low oxygen tension. *PLoS One* **10**, e0142724.

Kim J, Lee J, Kim SH and Jun JH (2016). Coculture of preimplantation embryos with outgrowth embryos improves embryonic developmental competence in mice. *Reprod Sci* **23**, 913–23.

Kleijkers SHM, van Montfoort APA, Bekers O, Coonen E, Derhaag JG, Evers JLH and Dumoulin JCM (2016). Ammonium accumulation in commercially available embryo culture media and protein supplements during storage at 2-8°C and during incubation at 37°C. *Hum Reprod* **31**, 1192–9.

Koscinski I, Merten M, Kazdar N and Guéant JL (2018). Culture conditions for gametes and embryos: which culture medium? Which impact on newborn? *Gynecol Obstet Fertil Senol* **46**, 474–80.

Lee J, Lee H, Lee Y, Park B, Elahi F, Lee ST, Park CK, Hyun SH and Lee E (2017). *In vitro* oocyte maturation in a medium containing reduced sodium chloride improves the developmental competence of pig oocytes after parthenogenesis and somatic cell nuclear transfer. *Reprod Fertil Dev* **29**, 1625–34.

Li ZC, Gu RH, Lu XW, Zhao S, Feng Y and Sun YJ (2018). Preincubation with glutathione ethyl ester improves the developmental competence of vitrified mouse oocytes. *J Assist Reprod Genet* **35**, 1169–78.

Lin T, Lee JE, Oqani RK, Kim SY, Cho ES, Jeong YD, Baek JJ and Jin DI (2016). Tauroursodeoxycholic acid improves pre-implantation development of porcine SCNT embryo by endoplasmic reticulum stress inhibition. *Reprod Biol* **16**, 269–78.

Lin XY, Beckers E, McCafferty S, Gansemans Y, Szymanska KJ, Pavani KC, Catani JP, Van Nieuwerburgh F, Deforce D, De Sutter P, Van Soom A and Peelman L (2019). Bovine embryo-secreted microRNA-30c is a potential non-invasive biomarker for hampered preimplantation developmental competence. *Front Genet* **10**, 15.

Liu Z, Foote RH and Yang X (1995). Development of early bovine embryos in co-culture with KSOM and taurine, superoxide dismutase or insulin. *Theriogenology* **44**, 741–50.

Mani S and Mainigi M (2018). Embryo culture conditions and the epigenome. *Seminars in Reprod Med* **36**, 211–20.

Men H, Stone BJ and Bryda EC (2020). Media optimization to promote rat embryonic development to the blastocyst stage *in vitro*. *Theriogenology* **151**, 81–5.

Pang YW, Jiang XL, Wang YC, Wang YY, Hao HS, Zhao SJ, Du WH, Zhao XM, Wang L and Zhu HB (2019). Melatonin protects against paraquat-induced damage during *in vitro* maturation of bovine oocytes. *J Pineal Res* **66**, e12532.

Pantaleon M, Ryan JP, Gil M and Kaye PL (2001). An unusual subcellular localization of GLUT1 and link with metabolism in oocytes and preimplantation mouse embryos. *Biol Reprod* **64**, 1247–54.

Qu P, Wang Y, Zhang C and Liu E (2020a). Insights into the roles of sperm in animal cloning. *Stem Cell Res Ther* **11**, 65.

Qu PX, Luo SW, Du Y, Zhang YR, Song XJ, Yuan XT, Lin ZJ, Li YH and Liu EQ (2020b). Extracellular vesicles and melatonin benefit embryonic develop by regulating reactive oxygen species and 5-methylcytosine. *J Pineal Res* **68**, e12635.

Rossi G, Di Nisio V, Macchiarelli G, Nottola S A, Halvaei I, De Santis L and Cecconi S (2019). Technologies for the production of fertilizable mammalian oocytes. *Appl Sci* **9**, 17.

Sanches BV, Pontes JHF, Basso AC, Ferreira CR, Perecin F and Seneda MM (2013). Comparison of synthetic oviductal fluid and G1/G2 medium under Low-1 oxygen atmosphere on embryo production and pregnancy rates in Nelore (*Bos indicus*) cattle. *Reprod Domest Anim* **48**, e7–9.

Sekirina GG and Neganova IE (1995). The microenvironment created by non-blocking embryos in aggregates may rescue blocking embryos via cell-embryo adherent contacts. *Zygote* **3**, 313–24.

Shohael AM, Ali MB, Yu KW, Hahn EJ, Islam R and Paek KY (2006). Effect of light on oxidative stress, secondary metabolites and induction of antioxidant enzymes in Eleutherococcus senticosus somatic embryos in bioreactor. *Process Biochem* **41**, 1179–85.

Simonstein F, Mashiach-Eizenberg M, Revel A and Younis JS (2014). Assisted reproduction policies in Israel: a retrospective analysis of *in vitro* fertilization-embryo transfer. *Fertil Steril* **102**, 1301–6.

Takahashi M (2012). Oxidative stress and redox regulation on *in vitro* development of mammalian embryos. *J Reprod Dev* **58**, 1–9.

Thouas GA, Jones GM and Trounson AO (2003). The 'GO' system – a novel method of microculture for *in vitro* development of mouse zygotes to the blastocyst stage. *Reproduction* **126**, 161–9.

Yang B, Zhu W, Zheng Z, Chai R, Ji S, Ren G, Liu T, Liu Z, Song T, Li F, Liu S and Li G (2017). Fluctuation of ROS regulates proliferation and mediates inhibition of migration by reducing the interaction between DLC1 and CAV-1 in breast cancer cells. *In Vitro Cell Dev Biol Anim* **53**, 354–62.

Yang Y, Wang LQ, Chen C, Qi HB, Baker PN, Liu XQ, Zhang H and Han TL (2020). Metabolic changes of maternal uterine fluid, uterus, and plasma during the peri-implantation period of early pregnancy in mice. *Reprod Sci* **27**, 488–502.

Zhu BK, Walker SK and Maddocks S (2004). Optimisation of *in vitro* culture conditions in B6CBF1 mouse embryos. *Reprod Nutr Dev* **44**, 219–31.

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