Regenerative Therapy 29 (2025) 319–327

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Co-culture with adipose mesenchymal stem cells promotes Blastocyst formation and gene expression in embryos from aged mice

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ARTICLE INFO

Article history:
Received 30 November 2024
Received in revised form
6 March 2025
Accepted 23 March 2025

Keywords:
Co-culture
Zygotes
Blastocyst
Mesenchymal stem cells

ABSTRACT

Recent studies have highlighted the positive effects of co-culturing embryos with stem cells on embryo development in various mammalian systems. Stem cells secrete numerous factors, including cytokines, growth factors, and microRNAs, which promote embryo development. However, the impact of stem cells on the development of embryos derived from aged mice's oocytes remains poorly understood. This study evaluated the co-culture effects of adipose tissue-derived mesenchymal stem cells (ADMSCs) on zygotes, focusing on the developmental potential of fertilized embryos. Embryo quality was assessed through staining techniques to measure trophectoderm (TE), inner cell mass (ICM), and total blastocyst cell numbers during *in vitro* culture. Results demonstrated that ADMSC co-culture significantly improved zygote cleavage and blastocyst development rates, particularly in embryos derived from aged mice. Enhanced implantation and post-implantation potential were observed in embryos from both young and aged mice. Notably, co-culture increased TE, ICM, and total blastocyst cell numbers in aged mice-derived embryos without inducing apoptosis in blastocysts. Gene expression analysis revealed upregulation of *OCT4 and G6PDH*, associated with pluripotency and glucose metabolism, particularly in embryos from aged mice, while the heat stress marker HSP70 showed no significant changes. These findings demonstrate the potential of ADMSC co-culture as a beneficial protocol for improving embryo development. These findings from this study could offer an important basis for future mechanistic studies in this area.

1. Introduction

The increasing global trend of delayed childbearing has heightened the need to better understand the causes and consequences of reproductive aging which is a key factor contributing to reduced fertility and adverse pregnancy outcomes [1]. Reproductive aging is associated with diminished oocyte quality, impaired fertilization, and compromised embryonic development, posing significant challenges for women seeking to conceive later in life. Data from the 1999 Assisted Reproductive Technology Success

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.
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https://doi.org/10.1016/j.reth.2025.03.017

Rates (ARTSR) report underscores the impact of age on reproductive outcomes. The rate of clinical pregnancies failing to result in live births shows a clear age-related trend, with a failure rate of 14 % among women under 35, 19 % for those aged 35–37, 25 % for ages 38–40, and a striking 40 % for women over [2]. These statistics emphasize the urgent need for novel strategies to mitigate the effects of reproductive aging and improve outcomes.

A primary factor contributing to the decline in fecundity with advancing age is believed to be the gradual loss of ovarian follicles, which begins at birth and culminates in the depletion of oocytes at menopause. Additionally, oocyte quality has been reported to deteriorate with age. In contrast, the uterus is thought to retain its capacity to sustain pregnancy for a significantly longer period, even as ovarian function declines [3–6]. The decline of oocyte quality is defined based on various criteria, including higher levels of DNA damage [7], decreased fertilization rate [8], delayed pronuclear formation [9] and abnormal calcium oscillations [10]. Chromosome aneuploidy has been identified as another major cause of age-related decline in oocyte quality [11,12].

In a previous study on the reproductive potential of aged mice, the mean number of recovered oocytes per mouse in the young group (8–11 weeks old) was significantly higher than that of the aged groups (40–49 weeks and 50–59 weeks old). Additionally, the rates of blastocyst formation and post-implantation embryo development (82.6 % at the early egg cylinder stage) in the young group were markedly higher than aged counterparts. Improvement of the quality of *in vitro* blastocysts from advanced maternal ages remains an important clinical issue. For successful *in vitro* production of mammalian embryos is a rich microenvironment for oocyte and embryo culture that effectively mimics the conditions within the reproductive tract [13,14]. While significant advances have been made in the design of defined and semi-defined embryo culture media [15–17], medium additives and somatic cell-based co-culture systems require optimization to obtain higher yields of better quality embryos [18–21]. Over recent years, considerable research interest has focused on the development of a novel approach based on the use of stem cells of adult or embryonic origin and their derived biomaterials in co-culture systems with oocytes and/or embryos from mice, porcine and bovine sources [22–25]. Although the recognition is not consistent until now that the utility of conditioned medium versus co-culture with stem cells, data from the above studies provide clear evidence of the beneficial effects of stem cell-based culture conditioning on *in vitro* embryo development. Here, we evaluated the potential benefits of mouse adipose tissue-derived mesenchymal stem cells (m-ADMSCs) either in co-culture or by conditioning culture medium in a mouse *in vitro* culture system. The ADMSCs was used in this study due to their special qualities, which include their high proliferation rate, low immunogenicity, accessibility, and well-established supporting functions in a variety of co-culture settings, as shown in previous studies [26,27].

This study examined the effects of co-culturing zygotes with adipose tissue-derived mesenchymal stem cells (ADMSCs) on embryo development. ADMSC co-culture significantly improved zygote cleavage, blastocyst development, and implantation potential. It increased trophectoderm (TE), inner cell mass (ICM), and total blastocyst cell numbers without causing apoptosis. Gene expression analysis showed higher levels of pluripotency (OCT4) and glucose metabolism (G6PDH) markers in aged mice-derived embryos, while the heat stress marker (HSP70) remained unaffected. These findings suggest that ADMSC co-culture could be a promising approach to enhance embryo development.

The structure of this research is designed to provide a comprehensive and systematic overview of the study. It begins with an introduction, encompassing a detailed literature review and background information. This is followed by a materials and methods section, which provides details information about the reagents, equipment, and experimental design. The results section subsequently presents the findings, accompanied by tables, graphs, and microscopy images. Finally, the discussion section interprets the results, highlighting their theoretical implications.

2. Materials and methods

2.1. Animals and collection of blastocysts in vitro

ICR virgin albino mice were housed under a controlled 12-h light/dark cycle and provided with food and water *ad libitum* in accordance with the guidelines of animal care and Use of Experimental Animals (Canadian Council on Animal Care, Ottawa, 1993; ISBN: 0-919087-18-3). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Pingtung University of Science and Technology (**Approval No. NPUST-110–120**). Female mice, aged 6–8 weeks, were maintained for a period of 40–52 weeks to simulate age-related reproductive conditions.

To induce superovulation, the mice were administered an intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (PMSG; 80056–608; VWR Scientific Inc.), followed 48–52 h later by an intraperitoneal injection of 7.5 IU human chorionic gonadotropin (hCG; CG-10; Sigma). Approximately 15–17 h post-hCG administration, superovulated mature female mice were sacrificed, and oocytes were collected. The oviducts were excised and transferred to liquid paraffin within a fertilization dish (35 mm × 10 mm, Cat. No. 430588; Corning). Using a dissecting needle, the ampullae of the oviducts were punctured to release cumulus-oocyte complexes (COCs). These COCs were incubated in a humidified environment at 37 °C with 5 % CO_2 in air for 30–60 min before insemination.

Mature male mice (10 weeks old) were sacrificed, and their cauda epididymides were excised and placed in liquid paraffin within a sperm preparation dish. Spermatozoa were released from the cauda epididymides into PERIUP® pre-incubation medium (Cosmo Bio Ltd.) and capacitated by incubation at 37 °C with 5 % CO_2 in air for 60 min. Approximately 3 μL of the capacitated sperm suspension was added dropwise to CARD Medium (Cosmo Bio) containing the prepared COCs, using a fine pipette tip (Cat. No. 114; Quality Scientific Plastic).

Following insemination, the fertilization dishes were incubated at 37 °C with 5 % CO_2 in air. Six hours post-insemination, oocytes were transferred to Human Tubal Fluid medium (mHTF) for observation. Parthenogenetic oocytes with a single pronucleus and normally fertilized oocytes were recorded. Mature oocytes were fertilized in groups of 10 COCs per 50 μL drop under oil immersion, using spermatozoa at a final concentration of 1×10^6 sperm/mL. The gametes were co-incubated for 6 h to facilitate fertilization.

Presumptive zygotes (10 per 20 μL drop under oil immersion) were then cultured in sequential media. Cleavage was assessed 24 h post-insemination, with non-cleaving or fragmented embryos removed from culture. Cleaved embryos were transferred to step 2 medium after 48 h of culture, and blastocysts were collected following 96–116 h of incubation.

2.2. Preparation and culture of adipose tissue-derived mesenchymal stem cells

Adipose tissue-derived mesenchymal cells (ADMSCs) were obtained from abdominal adipose tissues of female mice. Adipose tissue samples were dissociated into small clumps using scissors and rinsed with phosphate buffered saline (PBS). Minced adipose tissues (1: 1 mixed with 1 % collagenase (Gibco) were incubated for 1 h at 37 °C

followed by centrifugation at 300×*g* for 5 min. The resulting pellets were washed three times with Dulbecco's modified Eagle's high glucose medium (DMEM) supplemented with 10 % fetal bovine serum, 1 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). The pellet was suspended in DMEM and plated in culture dishes supplemented with DMEM in 5 % CO_2 at 37 °C. After 48 h, culture medium containing non-adherent cells was removed and adherent cells grown on culture dishes until confluence (10–12 days). During cell culture, ADMSCs displayed a fibroblast-like spindle-shaped morphology. The medium was replaced three times a week and all experiments performed on passage 4 or 5 cells.

For ADMSC characterization, culture-expanded cells were incubated with FITC- conjugated antibody at 4 °C for 30 min and washed twice in PBS supplemented with 2 % FBS. Cells were analyzed using a digital fluorescence-activated cell sorter (FACS) Canto II flow cytometer as described previously (Chen et al., 2016). Notably, ADMSCs were positive for CD90 and CD105 but negative for CD34. To obtain ADMSCs with high purity, cells were magnetically labeled with Microbeads and sorted via magnetic activated cell sorting (MACS) technology (Miltenyi Biotec). To this end, cell suspensions were incubated with magnetically labeled anti-CD90 and anti-CD105 antibodies and loaded onto MACS columns placed in the magnetic field of the separator. Cells with magnetically labeled CD90 and CD105 were retained within the column.

After removing the column from the separator, magnetically retained CD90 and CD105-positive cells were eluted as the ADMSC fraction and subsequently sorted and expanded for further experimental use. The stem cell potential of ADMSCs was further confirmed by inducing differentiation into chondrocytes, osteocytes and adipocytes [28]. Prior to embryo co-culture, ADMSCs were inactivated by treatment with 10 μg/mL mitomycin C (Sigma-Aldrich) for 2 h and rinsed three times with PBS. Inactivated ADMSCs were maintained in DMEM overnight. Preparation of inactivated ADMSCs in drop culture was modified according to a previous report [23].

2.3. Zygotes and co-culture with ADMSCs

Mature oocytes were fertilized (10 COCs per 50 μL drop under oil immersion) using spermatozoa from males (final concentration of 1 x 10^6 sperm/mL). The gametes were co-incubated for 6 h. After *in vitro* fertilization (IVF), presumptive zygotes (10 per 20 μL drop under oil immersion) were collected for subsequent experiments.

Zygotes obtained from IVF were washed with KSOM medium for three times and morphologically normal zygotes randomly cultivated in 50 μL KSOM medium under mineral oil in all experimental conditions. At 24 h before IVF, inactivated ADMSCs were trypsinized and 10^4 cells were resuspended in 50 μL KSOM for acclimatization. Drops (25 μL) of conditioned medium from ADMSCs were plated with 25 μL KSOM. Embryonic development was evaluated daily for 4 days after incubation with KSOM or co-culture with inactivated ADMSCs or ADMSC-conditioned medium (mixed with KSOM, 1:1). Cleavage, morula and blastocyst formation abilities were evaluated by counting after 4 days of culture and the percentages calculation. Values are presented as means ± SD of three to four determinations.

2.4. Blastocyst culture and definition of developmental stages in vitro

Blastocysts culture *in vitro* was based on modification of a previously reported method [29]. In brief, embryos were cultured with CMRL-1066 medium supplemented with 1 mM glutamine and 1 mM sodium pyruvate plus 50 IU/mL penicillin and 50 mg/mL streptomycin in 4-well multi-dishes at 37 °C. Thereafter, embryos were cultured for 3 days in medium supplemented with 20 % fetal calf serum and 4 days in medium supplemented with 20 % heat-inactivated human placental cord serum for a total time of 8 days from the onset of treatment.

Embryos were inspected daily under a phase-contrast dissecting microscope and developmental stages classified according to established guidelines [30]. Developmental parameters, such as hatching through the zona pellucida, attachment to the culture dish, trophoblastic outgrowth and differentiation of the embryo proper into early or late egg cylinders (germ layer stage) or primitive streak to the early somite stage, were recorded daily. To decrease observer bias, all differentiation stages of mouse embryos *in vitro* were classified according to the guidelines [30]. Implanted blastocysts were classified based on attachment and outgrowth of blastocysts to culture dishes. An early egg cylinder (EEC) embryo was defined as an embryo that had reached stage 9 or 10 by day 4 and late egg cylinder (LEC) embryo as one that had reached stage 11, 12 or 13 by day 6 of culture. An embryo that had reached stage 14 or 15 by day 8 was classified as an early somite stage (ESS) embryo. Values are presented as means ± SD of six to eight determinations.

2.5. Cell proliferation of in vitro blastocysts co-cultured with stem cells

In vitro blastocysts collected from IVF with or without stem cell co-culture were used to evaluate proliferation via the differential staining method. Blastocyst proliferation was assessed by separately counting the number of inner cell mass (ICM) and trophectoderm (TE) cells identified via dual differential staining. In brief, the zona pellucida of embryos was removed by exposure to 0.4 % pronase solution in Earle's Balanced Salt Solution (EBSS; Sigma)-BSA medium containing 0.3 % bovine serum albumin (BSA) at 37 °C for 5 min. Denuded blastocysts were incubated with 15 % rabbit anti-mouse serum at 37 °C for 30 min and subsequently washed with EBSS-BSA medium. Embryos were further reacted with 10 % bovine serum, 2 mg/mL bisbenzimide, and 1 mg/mL propidium iodide at 37 °C for 30 min. The resulting immunolysed blastocysts were gently transferred to chamber slides under careful protection from light. Under appropriate UV light excitation, ICM cells that take up bisbenzimide but exclude propidium iodide are stained blue whereas TE cells labeled with both fluorochromes are stained orange-red. The numbers of nuclei were calculated as an accurate reflection of the cell number.

2.6. Analysis of apoptosis of in vitro blastocysts co-cultured with stem cells

Apoptosis of *in vitro* blastocysts collected from IVF with or without stem cell co-culture was evaluated using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. In brief, embryos were washed with embryo culture medium three times and fixed with 4 % paraformaldehyde (PFA) at room temperature for 2 h, followed by incubation with 20 μL TUNEL reaction mixture (2 μL enzyme solution plus 18 μL labeling solution containing fluorescein-conjugated nucleotides) at 37 °C. After reaction for 30 min, embryos were washed three times with PBS containing 0.3 % (w/v) BSA and further incubated with converted POD solution (20 μL) for 30 min at 37 °C. Finally, embryos were incubated with 20 μL of 3,3′-diaminobenzidine (DAB) substrate solution for 2 min and apoptotic cells assessed via fluorescence microscopy. TUNEL-positive cells representing the apoptotic population were detected as black spots and counted under a light microscope.

2.7. Quantitative real-time PCR analysis of gene expression

The mRNA expression levels of OCT4, G6PDH and HSP70 were measured via quantitative real-time PCR. Briefly, total RNA of

embryos was isolated and purified using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. The mRNA levels were quantified with the ABI 7000 Prism Sequence Detection System (Applied Biosystems, CA, USA) and normalized to β-actin mRNA as the internal endogenous control. All experiments were conducted three times in triplicate and the average values used for quantification. The sequences of primers used for real-time quantitative RT-PCR were as follows: OCT4 (Forward 5'-CCT GGG CGT TCT CTT TGG AAA GGT G-3' and Reverse 5'- GCC TGC ACC AGG GTC TCC GA-3'), G6PDH (Forward 5'- CAT CAC CCT GGT ACA ACT CTT-3' and Reverse 5'-CGG CAA CTA AAC TCA GAA AAC-3'), HSP70 (Forward 5'-GAA GGT GCT GGA CAA GTG C-3' and Reverse 5'-GCC AGC AGA GGC CTC TAA TC-3') and β-actin (Forward 5'-CGT ACC ACA GGC ATT GTG ATG-3' and Reverse 5'-CTT CTA GGA CTG GCT CGC AC-3').

2.8. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Dunnett's test for multiple comparisons with IBM SPSS software version 26. Data are expressed as means ± standard deviation (SD) and statistical significance differences considered at $p < 0.05$.

3. Results

3.1. Effects of co-culture with ADMSCs on pre-implantation embryo development in vitro

The mean number of oocytes collected per mouse was significantly higher in young mice compared to aged mice (Table 1). Additionally, the *in vitro* fertilization (IVF) rate was greater in the young group than in the aged group (Table 1). To evaluate the potential effects of adipose-derived mesenchymal stem cells (ADMSCs) on embryonic development, fertilized zygotes from both young and aged groups were co-cultured with ADMSCs, and their *in vitro* developmental progress was analyzed. In young mice, zygote cleavage rates to the 2-cell stage and subsequent development to the blastocyst stage were significantly higher in the ADMSC co-culture group than in the non-co-culture group (Table 2). For aged mice, the co-culture group exhibited markedly higher rates of zygote development at all observed stages *in vitro,* including the 2-cell, 4- to 8-cell, 16- to 32-cell, and blastocyst stages, compared to the non-co-culture group (Table 2). Notably, the improvement in embryonic development rates resulting from ADMSC co-culture was more pronounced in aged mice than in young mice.These findings suggest that co-culturing mouse zygotes with ADMSCs significantly enhances cleavage and blastocyst formation rates.

3.2. Effects of co-culture with ADMSCs on post-implantation embryo development in vitro

To further establish the effects of co-culture with ADMSCs on post-implantation events *in vitro*, zygotes were cultured with or without ADMSCs and *in vitro* development for 8 days, analyzed. Zygotes of both young and aged groups co-cultured with ADMSCs showed a higher incidence of post-implantation developmental milestones (Table 3). In the young age group, the rates of embryo implantation and attachment to fibronectin-coated cultured dishes were significantly higher in the ADMSC co-culture than non-cultured control group (Table 2). In addition, embryos of the ADMSC co-culture group formed two-layer ICM and ectoplacental cones at a higher rate and showed more instances of embryonic development to advanced egg cylinder stages (LEC and ESS) relative to the control group (Table 3).

Moreover, embryos of aged groups co-cultured with ADMSCs showed a significantly higher rate of development to the early egg cylinder (EEC) than non-cultured control group (Table 3). Stage-specific effects were observed, with significant improvements in the Aging-ADMSC group at the early egg cylinder stage (EECS) and in the Young-ADMSC group at the late egg cylinder (LECS) and early somite stages (ESS).

3.3. Effects of co-culture with ADMSCs on cell proliferation potential and apoptosis during oocyte-to-blastocyst maturation in vitro

The effects of co-culture with ADMSCs on embryo proliferation were analyzed by assessing trophectoderm (TE), inner cell mass (ICM) and total blastocyst cell numbers during IVM of zygotes cultured with or without ADMSCs using the differential staining method (using Hoechst and propidium iodide (PI) (Fig. 1A). Co-culture of aged group zygotes with ADMSCs induced a marked increase in TE, ICM, and total cell numbers during IVM compared with the KSOM culture (control) group whereas no significant effects were observed on co-cultured zygotes of young age groups (Fig. 1B). TUNEL staining was further employed to measure apoptotic cells in

Table 1
Comparison of oocyte yield and *in vitro* fertilization efficiency between young and aged mice.

	No. of oocytes	Mean number of oocytes	IVF-zygote	Fertilization rate
Young (n = 11)	528	40.62*	461	87.31 %* (461/528)
Aging (n = 38)	208	5.47	159	76.44 % (159/208)

A statistically significant difference was observed in the fertilization rates between the two groups, with the young group showing a significantly higher rate (*p < 0.05) compare to age group. These results emphasize a marked decline in reproductive performance with age, evident in both the quantity and quality of oocytes retrieved and their ability to undergo successful fertilization *in vitro*.

Table 2
Effects of co-culturing oocytes from aging or young mice with ADMSCs on pre-implantation embryo development *in vitro*.

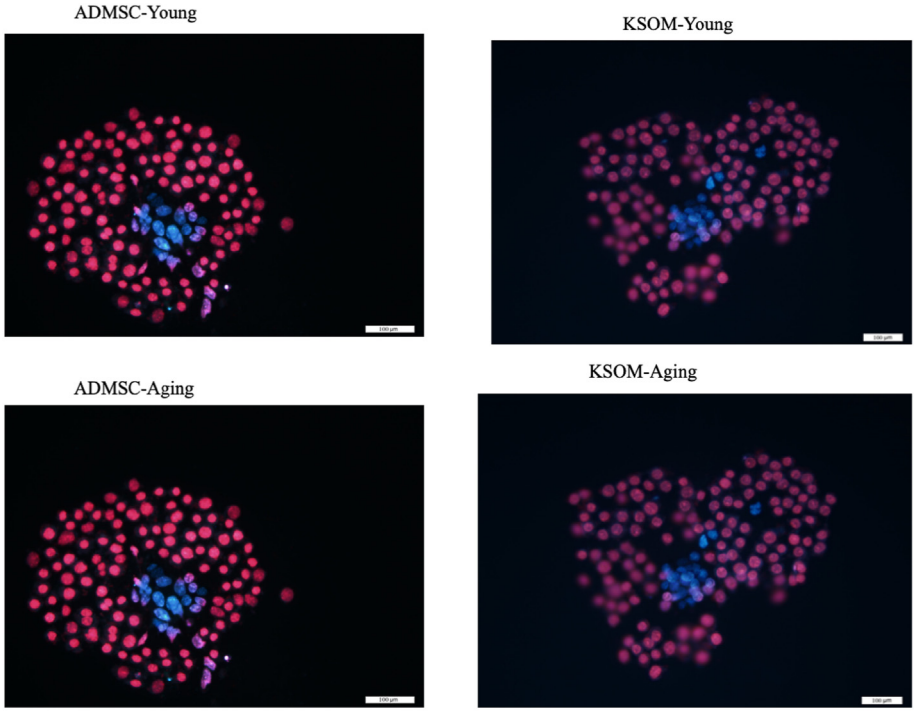
	Zygote	2-cell embryo	4 to 8-cell embryo	16 to 32-cell embryo	Blastocyst
Y-ADMSC	78	92.31 % (72/78)	88.46 % (69/78)	83.33 %* (65/78)	82.05 %* (64/78)
Y-KSOM	116	82.76 %* (96/116)	79.31 % (92/116)	70.69 % (82/116)	61.21 % (71/116)
A-ADMSC	68	94.11 %* (64/68)	92.65 %** (63/68)	88.24 %** (60/68)	85.29 %*** (58/68)
A- KSOM	67	83.58 % (56/67)	71.64 % (48/67)	68.66 % (46/67)	64.18 % (43/67)

Here, Y-ADMSC and A-ADMSC belong to Oocytes from young (Y) and aging (A) mice co-culture with ADMSC while Y-KSOM and A-KSOM belong to Oocytes from young (Y) and aging (A) mice culture with KSOM. Here * refers to a significant difference, $p < 0.05$, ** refers to a significant difference, $p < 0.01$, ***refers to a significant difference, $p < 0.001$, compare to non-culture group).

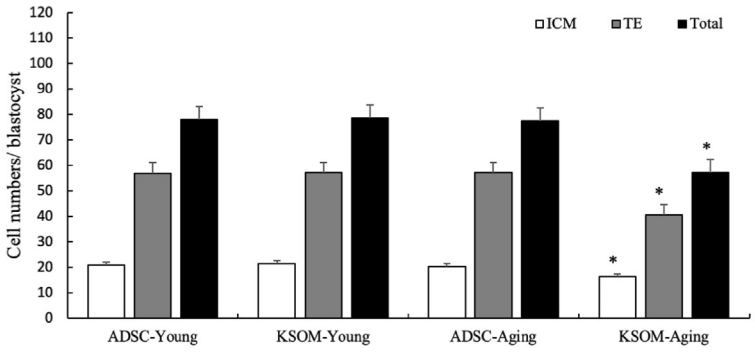
Table 3
In vitro development of mouse zygotes co-cultured with ADMSCs.

Development	Young		Aging	
	ADMSC	KSOM	ADMSC	KSOM
Blastocysts	75	68	41	29
Hatched/implanted blastocysts	93.33 %* (70/75)	100 % (68/68)	90.24 % (37/41)	93.10 % (27/29)
Early egg cylinder stage (EECS)	85.33 % (64/75)	86.77 % (59/68)	73.17 %* (30/41)	58.62 % (17/29)
Late egg cylinder stage (LECS)	49.33 %*** (37/75)	22.06 % (15/68)	29.27 % (12/41)	34.48 % (10/29)
Early somite stage (ESS)	26.67 %** (20/75)	10.29 % (7/68)	24.39 % (10/41)	24.14 % (7/29)

Here KSOM refers to the Control group.
Here * refers to a significant difference, $p < 0.05$, ** refers to a significant difference, $p < 0.01$, ***refers to a significant difference, $p < 0.001$, compare to non-culture group).



(A)



(B)

Fig. 1. Effects of co-culturing mouse zygotes with ADMSCs on embryo proliferation during *in vitro* development from the zygote to blastocyst stage. Zygotes from both young and aged groups were co-cultured with ADMSCs for 4 days, and blastocyst development was assessed. Cells of the inner cell mass (ICM) and trophectoderm (TE) were differentially stained using Hoechst and propidium iodide (PI) (A). counted (B) via differential staining. Data from 180 blastocysts were obtained for each group. Values are presented as means ± SD of five to six determinations. (* indicates a significant difference, $P < 0.05$, compared to the control group). Here KSOM refers to control group.

blastocysts derived from oocytes co-cultured *in vitro* with ADMSCs. Subsequent quantitative analyses revealed no significant differences in apoptosis among the young and aged co-culture groups relative to the KSOM culture (control) group (Fig. 2). Our findings indicate that co-culture with ADMSCs promotes proliferation specifically in embryos from aged mice but has no significant effects on embryos from young mice. In addition, zygotes co-cultured with ADMSCs showed detrimental effects in terms of triggering apoptosis in both young and aged groups of mice.

3.4. Effects of co-culture with ADMSCs on gene expression during oocyte to blastocyst maturation in vitro

The effect of co-culture with ADMSCs on embryo development quality was determined based on gene expression analysis using real-time PCR (RT-PCR). Co-culture of zygotes with ADMSCs during development to the blastocyst stage led to a significant increase in the expression levels of pluripotency (OCT4) and glucose metabolism (G6PDH) markers relative to the corresponding gene levels in KSOM culture groups from both young and aged mouse groups (Fig. 3A and B). The magnitude of increase in the Aging-ADMSC group was more pronounced, particularly for OCT4, suggesting a stronger response in aged mice (Fig. 3A). However, expression of the heat stress marker, HSP70, was not affected by ADMSCs co-culture (Fig. 3C). The overall findings indicate enhancement of the quality of embryos obtained from co-culture with ADMSCs during *in vitro* development.

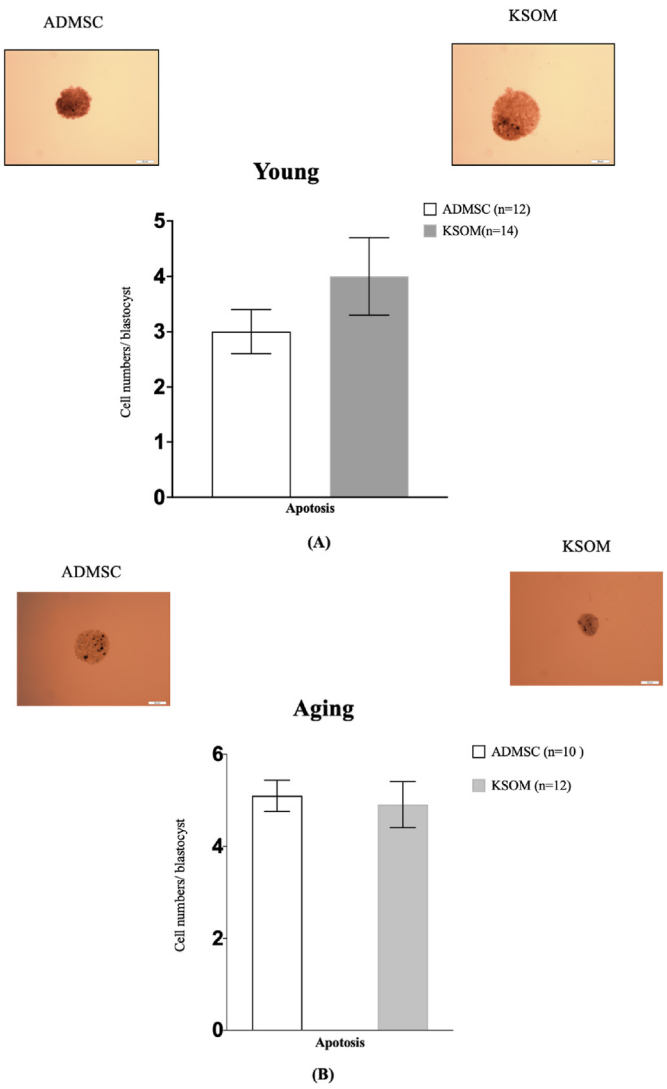


Fig. 2. Effects of co-culturing mouse zygotes with ADMSCs on cell apoptosis in blastocyst-stage embryos. Zygotes of both young and aged groups were co-cultured with ADMSCs for 4 days and allowed to develop into blastocysts *in vitro*. (A) Apoptosis of blastocysts was detected via TUNEL staining. (B) Apoptosis-positive cells were observed as black spots under light microscopy and counted per blastocyst. Data from 150 blastocysts were analyzed for each group. Values are presented as means ± SD of three to five determinations.

4. Discussion

In this study, we have demonstrated for the first time significant beneficial effects of co-culture of zygotes with ADMSCs relative to KSOM on the rate of zygote cleavage and development to blastocysts, implantation and post-implantation *in vitro* development, cell proliferation, and embryo quality, especially in oocytes from aged mice. Our findings are consistent with earlier data showing favorable effects of combining embryo cultures with stem cells [31,32]. Previously Moshkdanian et al. [24] reported that co-culture of mouse embryos with human umbilical cord mesenchymal cells (h-UCMS) improved the development potential of embryos under conditions of light stress. In addition, co-culture of bovine embryos with bovine adipose tissue-derived mesenchymal cells (b-ATMSCs) had a more significant beneficial effect than a traditional co-culture system with granulosa cells, highlighting the advantage of culturing embryos with stem cells from the same species [23]. Data from the current study further confirm improvement of the pre- and post-implantation embryo development potential of zygotes upon co-culturing with stem cells.

The effects of co-culturing with stem cells on embryo development are likely to be attributed to paracrine modulation by the microenvironment [23]. Several investigations have shown that mesenchymal stem cells exert beneficial or positive effects on immunomodulation, angiogenesis, and/or tissue homeostasis *in vitro* and *in vivo* [33–35]. Given the collective results from previous and current experiments, development of a stem cell-free model for therapy may be a desirable approach in the future, since using stem cell-conditioned medium could also exert positive effects on embryo development, avoiding cell membrane-related antigen reactions and providing interspecies therapeutic opportunities. In addition, cell-conditioned medium for cell therapy or embryo development is more effective in controlling therapeutic quality for clinical and commercial applications than co-culture with stem cells.

Several studies have reported that growth factors, cytokines, and microRNAs contained and secreted by exosomal microvesicles exert beneficial effects and potentially serve as effector molecules for cell therapy studies, and co-culturing could significantly promote stem cell differentiation for tissue regeneration [33,34,36]. Numerous cell types secrete small exosomal vesicles (60–200 nm) into extracellular fluids and culture medium, including MSCs. These vesicles contain mRNAs, microRNAs, and even proteins that are delivered to target cells through fusion with the plasma membrane [37–40]. Recent studies suggest that MSC-derived exosomal microvesicles have micromodulation potential in stem cell therapy or co-culture applications. In addition, preimplantation embryos of several species, including human, mice and cattle, secrete small exosomal vesicles throughout the *in vitro* culture periods [41–43]. Collective data from both previous and current studies support a potential reciprocal effect of ADMSCs and zygotes or developmental embryos in culture that could explain the observed effects in our experiments. However, the detailed regulatory mechanisms and precise roles of exosomes require further investigation.

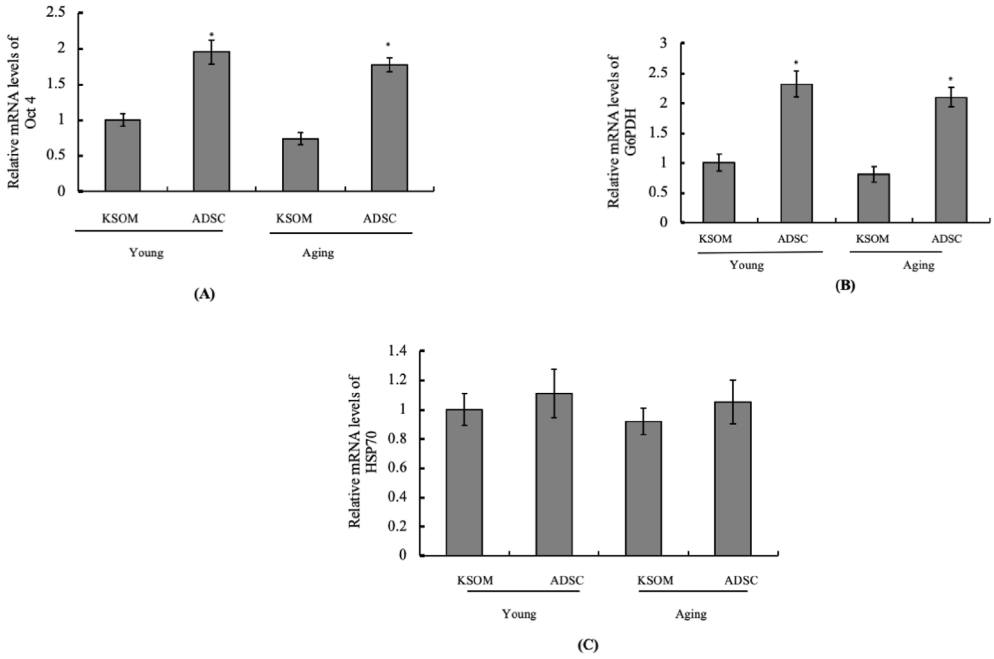


Fig. 3. Effects of co-culturing mouse zygotes with ADMSCs on gene expression in blastocyst-stage embryos. Zygotes of both young and aged groups were co-cultured with ADMSCs for 4 days and allowed to develop into blastocysts *in vitro*. OCT4 (A), G6PDH (B) and HSP70 (C) mRNA expression levels were analyzed via real-time RT-PCR. Data from 120 blastocysts were analyzed for each group. Values are presented as means ± SD of three to five determinations. (* indicates a significant difference, $P < 0.05$, compared to the control group). Here KSOM refers to control group.

Notably, results from gene expression analysis showed that co-culturing with ADMSCs not only promoted embryo development from zygotes into blastocysts and post-implantation development potential but also improved embryo developmental quality, especially in the aged mouse groups. Embryos co-cultured with ADMSCs showed a significant increase in OCT4/POU5F1 expression than those cultured in KSOM medium (control group). OCT4/POU5F1 is an important transcriptional regulator necessary for the maintenance of pluripotency potential in mammal embryonic stem cells and developing embryos [44]. In addition, G6PDH gene expression was upregulated in co-cultures with ADMSCs. G6PDH acts as a key regulator of NADPH availability for fatty acid and/or steroid biosynthesis and carbohydrate anabolic or catabolic reactions and promotes generation of ribose 5-phosphate involved in nucleotide and nucleic acid synthesis [45,46]. Both OCT4 and G6PDH genes are good indicators of embryo development quality [47–51]. In our experiments, co-culture with ADMSCs increased embryo developmental potential and quality from both young and aged mouse groups. Notably, this increase was more significant in embryos of fertilized oocytes obtained from aged mice.

In summary, co-culture of embryos with ADMSCs not only increased pre- and post-implantation embryo developmental potential but also improved energy metabolism of embryos (as indicated by G6PDH) but did not affect cellular stress (as indicated by HSP70), which could explain the significant increase in pluripotency marker OCT4 in ADMSC co-culture groups. These findings support the utility of co-culture with MSCs as a potentially effective protocol for *in vitro* embryo development with the *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) artificial reproduction systems. However, the regulatory mechanisms and key molecules produced by MSCs during co-culturing that are involved in stimulation of embryo development require further investigation. In addition, future studies should focus on implantation and post-implantation development of embryos co-cultured with ADMSCs *in vivo* via embryo transfer.

5. Conclusions

This study demonstrates the stage-specific benefits of ADMSC co-culture on embryo implantation and development. Aging-ADMSCs showed significant effects at the expanded early cavitation stage (EECS), while Young-ADMSCs were most effective during the late expanded cavitation stage (LECS) and expanded spherical stage (ESS). Co-culture significantly enhanced zygote development to the blastocyst stage, with more pronounced improvements in aged mice compared to young mice. Post-implantation development, cell proliferation, and embryo quality were markedly improved in the co-culture groups, outperforming conventional media like KSOM. The enhanced outcomes were supported by increased expression of pluripotency marker *OCT4* and metabolic regulator *G6PDH*, while maintaining stable *HSP70* levels, indicating no cellular stress. These findings underscore the potential of ADMSC co-culture to improve cleavage rates, blastocyst formation, and post-implantation potential. However, the study highlights the need for further investigation into the mechanisms by which ADMSCs influence development and the bioactive factors involved. While OCT4, G6PDH, and HSP70 were used to assess pluripotency, metabolism, and stress response, future studies should include additional markers for a more comprehensive understanding. Additionally, *in vivo* implantation and post-implantation outcomes remain unexplored. Future research should address these limitations and focus on translating ADMSC co-culture benefits into clinical applications.

CRediT authorship contribution statement

Yan-Der Hsuuw: Conceptualization, Writing - Original Draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Yu-Ting Su: Conceptualization, Writing - Original Draft, Writing - review & editing, Supervision.

Wen-Hsiung Chan: Writing - review & editing, Visualization.

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All authors participated in the analysis and interpretation of the data, contributed to the development of the manuscript, and approved its final version.

Funding

This work was supported by grants from the NPUST-KCGMH joint research project (CORPG8L0341), Chang Gung Memorial Hospital, Taiwan.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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