Reproductive Biology and Endocrinology

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# Cell-free fat extract improves embryo development and clinical outcomes in older women with previous in-vitro fertilization failure

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## Abstract

**Background** Preimplantation embryos in vivo are exposed to various growth factors in the female reproductive tract that are absent in in vitro embryo culture media. Cell-free fat extract exerts antioxidant, anti-ageing, and ovarian function-promoting effects. However, its effects on embryo quality are yet to be investigated.

**Methods** We assessed the effect of cell-free fat extract supplementation on embryo culture using a naturally ageing mouse model. We assessed the model's efficacy in influencing embryo development and pregnancy rates in older women with in vitro fertilization failure. In addition, we performed immunofluorescence staining, multiplex immunoassay, whole-genome amplification and DNA sequencing, time-lapse embryo monitoring, and in vitro experiments.

**Results** Cell-free fat extract-supplemented media has a suitable osmolarity and pH and contains high levels of bioactive growth factors. Cell-free fat extract promoted embryo development and implantation in aged mice, probably by increasing embryo growth rate, inhibiting cell apoptosis, and promoting blastocyst adhesion. Clinical results showed that the cell-free fat extract group had significantly higher rates of the day 3 available and high-quality embryos than the control group, and the rate of usable embryos tended to be higher in the cell-free fat extract group. Furthermore, implantation and clinical pregnancy rates improved in the cell-free fat extract group than in the control group.

**Conclusions** Our study implies that cell-free fat extract supplementation can promote embryo development and clinical outcomes and may serve as a rescue strategy for older women with in vitro fertilization failure.

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**Keywords** Embryo development, Culture media, Cell-free fat extract, Growth factor, In vitro fertilization, Older women, Infertility

## Background

Women of advanced reproductive age are more likely to encounter recurrent poor-quality embryo morphology phenotypes and repeated in vitro fertilization (IVF) failures [1]. After embryo transfer, implantation and pregnancy rates remain low in women aged ≥ 35 years [2, 3]. Therefore, improving embryo development and clinical pregnancy outcomes in these patients is a challenge associated with assisted reproductive technology.

The composition of the embryo culture medium may affect the developmental potential of embryos generated during IVF cycles, influencing implantation and pregnancy rates [4]. Using unconditioned culture media containing embryonic trophic factors, such as cytokines and growth factors, for example, granulocyte-macrophage colony-stimulating factor (GM-CSF), heparin-binding epidermal growth factor (HB-EGF), leukaemia inhibitory factor (LIF), and insulin-like growth factor 1 (IGF1) results in better preimplantation embryo development in *vitro* [5, 6]. In addition, reactive oxygen species (ROS) are generated during in vitro embryo culture, resulting in elevated oxidative stress [7]. Therefore, supplementing the culture media with specific embryotrophic and antioxidant factors may support embryo quality better.

Cell-free fat extract (CEFFE) is a cell-free liquid with diverse, active proteins extracted from human subcutaneous adipose tissue through mechanical emulsification, centrifugation, and sterilisation. Proteomic analysis revealed that CEFFE contains over 1700 protein components, including EGF, basic fibroblast growth factor (bFGF), and many other growth factors similar to embryotrophic factors [8]. This suggests that CEFFE is a new promising bioactive substance. Furthermore, CEFFE is advantageous because it has an autogenous source, low immunogenicity, high clinical application safety, and can be stored with high activity at low temperatures for a long time. The antioxidant, anti-apoptotic, proliferative, and anti-ageing effects of CEFFE have shown promising results in research on endometrial regeneration, ischemic angiogenesis, and skin defects [9]. CEFFE alleviates osteoporosis by inhibiting ROS formation and reducing osteocyte apoptosis [10]. CEFFE partially promotes tissue repair by promoting angiogenesis and cell proliferation [9, 11]. Our previous study revealed that CEFFE ameliorated ovarian function and fertility when injected intravenously in mice of advanced age and those with primary ovarian insufficiency (POI), probably by improving granulosa cell function and ameliorating the ovary's overall microenvironment [12, 13]. Moreover, CEFFE is a liquid without cellular components, thus preventing the safety issues associated with cell-based therapies. In addition, safety evaluations suggested that CEFFE had no adverse effects on the offspring's growth and biosafety in mice and rats [12–14]. Therefore, we hypothesised that CEFFE supplementation in culture media might be particularly beneficial for the in vitro culture of embryos.

In this study, we explored the efficacy, safety, and underlying molecular mechanisms of CEFFE in embryo development using a naturally ageing mouse model. We also assessed the effects of CEFFE supplementation on embryo development in vitro and pregnancy rates in older women with infertility.

## Methods

### Chemicals and reagents

CEFFE was provided by SEME Cell Technology Co. As previously described, its protein concentration was adjusted to 3 mg/mL [13], and then subpackaged and frozen at − 80 °C for future experiments within 6 months. All other chemicals were purchased from Sigma Chemical Co., unless otherwise indicated.

### Ethics statement

The Ruijin Hospital Institutional Ethics Committee approved the study protocol (number: 2022 – 216). Participants provided written informed consent before participation.

### Mice embryo acquisition, culture, and time-lapse monitoring

C57BL/6 female mice aged 8 weeks or 10 months were injected with 10 IU pregnant mare serum gonadotropin and, 48 h later, with10 IU human chorionic gonadotropin to induce superovulation. Dose-response studies were performed using 8-week-old mice to select the best-fitting dose of CEFFE and 10-month-old mice to mimic women aged 35–40 years. The female mice were housed overnight with 8-week-old males. After 18 h, zygotes were collected from the ampullae, washed twice in glucose-minimum essential medium oxaloacetate pyruvate serum PLUS medium, and transferred to G1-Plus medium with different CEFFE concentrations (0, 1, 2, and 5%). Two days later, the embryos were transferred to the G2-Plus medium containing the different CEFFE concentrations (0, 1%, 2%, and 5%) referred to our previous in vivo research on both POI [12] and natural ageing mice model [13]. The time-lapse system comprised a commercially available incubator (Embryo Scope), with images recorded automatically every 20 min. We recorded the timing of embryo morphokinetic events,

including pronuclear fading, cleavage division from 2- to 8-cell stages, morula (merging of individual cells and reduction in embryo volume), cavitation (blastocoel appearance), blastocyst (blastocoel occupies half or more of embryonic volume), expanded blastocyst (the embryo is filled with the blastocoel), hatching blastocyst (initiation of hatching) and hatched blastocyst (hatched completely from the zona pellucida) [15].

#### Immunofluorescence analyses

To identify the trophoblast (TE) numbers, inner cell mass (ICM) numbers and total cell numbers of blastocysts obtained from different cultivation systems, GATA3 (TE marker), SOX2 (ICM marker) and 4', 6-Diamidino-2-Phenylindole (DAPI) were stained separately. Briefly, after mice embryo acquisition and grouping culture, hatched blastocysts were obtained, fixed in 2% paraformaldehyde for 0.5 h, and permeabilised with phosphate-buffered saline (PBS) + 0.3% Tween 20 + 0.2% Triton X 100 for 0.5 h. Blocking was performed in PBS, 2% bovine serum albumin and 30% goat serum for 1 h. The samples were incubated with a primary antibody (Rb anti-Sox2 #ab92494; Ms anti-GATA3 #ab282110; Abcam) overnight at 4 °C and a secondary antibody (anti-rabbit Alexa 594, # A-11012; anti-mouse Alexa 488, # A-11001; Life Technologies) for 2 h at 25 °C. The nuclei were stained with DAPI for 10 min at 25 °C. Fluorescence was visualised using a confocal microscope (Zeiss LSM 900; Zeiss, Germany).

#### Detection of apoptosis levels in mice embryos

Hatched blastocysts were collected for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (TUNEL) cell apoptosis detection (Beyotime, China), and cell apoptosis levels were analysed following the manufacturer's instructions. After permeabilization, the samples were incubated with deoxyuridine triphosphate-fluorescein isothiocyanate from the TUNEL kit for 1 h at 37 °C and apoptotic cells were labelled with green fluorescence. Then, the nuclei were stained with DAPI (Roche, Basel, Switzerland) for the total cell counting. The apoptotic rate was calculated using the formula: apoptotic cells/ total cells × 100%. Images were captured using a confocal microscope (TCS Sp8 STED; Leica).

#### In vitro implantation model using mouse blastocysts

The in vitro implantation model was constructed as previously described, with some modifications. During the mid-secretory phase of the endometrium, primary human endometrial epithelial cells (HEECs) were isolated from women with tubal obstruction factor-related infertility. Fresh endometrial biopsy samples were minced and digested using 1 mg/mL collagenase type I in a 37 °C shaker for 0.5 h. The mixture was sequentially passed through 100 μm and 40 μm sieves. After washing the 40 μm sieve with PBS, HEECs were obtained from the filtrate by centrifugation at 100 × g for 5 min and then seeded onto the collagen-coated 35 mm dish in 30 μL droplets of Dulbecco's modified eagle medium/F12 with 10% fetal bovine serum under paraffin oil and grown to confluence.

Hatched blastocysts from both groups were subsequently added to primary HEEC-confluent monolayers in the G2plus media. The co-cultured cells were incubated at 37 °C for 24 h, followed by two sets of washing with PBS. The adherent blastocysts were imaged by a fluorescence microscope (Nikon, TS, Japan). The co-cultures were fixed and permeabilised at room temperature as described above and stained with rhodamine-phalloidin (1:100) for F-actin and in DAPI for nuclei. We visualised signals using a confocal microscope (Leica TCS Sp8 STED).

#### Multiplex immunoassay

To analyse the growth factor concentrations in CEFFE supplementation, the "ProcartaPlex Human Growth Factor Panel" – immunoassay (EPX110-12170-901, eBioscience, Germany) for the Luminex platform was performed according to the manufacturer's instructions.

#### Participants and clinical study design

This prospective open-label study was performed at the Reproductive Medical Centre of Ruijin Hospital of Shanghai. Enrolment began from January 2022 through March 2023, with follow-up continuing until December 2023. The inclusion criteria were as follows: (i) age ≥ 35 years, (ii) a history of unsuccessful IVF cycles because of poor embryo quality (rate of useable embryos per fertilized oocyte is < 35% or blastocyst formation rate is < 25%), and (iii) the provision of signed informed consent. The exclusion criteria were as follows: (i) couples with known chromosomal aberration, (ii) endometriosis, (iii) adenomyosis, (iv) hydrosalpinx, (v) morphologically abnormal oocytes > 50%, or (vi) normal sperm morphology rate < 2%.

For the embryo culture stage, we included: (i) 22 patients with ≥ 6 retrieved oocytes using the sibling-oocyte split. The patients were randomly divided by allocation concealment into a control and a CEFFE group using a 1:1 allocation ratio as described [16]. Briefly, each patient's cohort of cumulus-oocyte complexes was split into two equivalent groups labelled A and B. This simple randomisation, which indicated either a control or CEFFE culture system was implemented using a random number generator. The self-control studies were performed in sibling oocytes. (ii) 34 patients with < 6 retrieved oocytes; intracytoplasmic sperm injection (ICSI) was performed and single cultured in a CEFFE-supplemented medium. Self-control studies were conducted in this cycle with

the previous cycle within 1 year using the same ovulation induction protocol. For the embryo transfer stage, 43 women were enrolled to receive thawed embryos from either the CEFFE treatment or control group (schematic illustration, Fig. 1).

### Stimulation protocol, IVF, and embryo quality assessment

This study's female participants followed progestin primed ovarian stimulation protocol or gonadotropin-releasing hormone antagonist protocol as described previously [17]. For oocytes retrieved from the controlled ovarian hyperstimulation cycle, the CEFFE group was supplemented using a sequential culture media (G-IVF plus, G1 plus, and G2 plus, supplied by Vitrolife) with the best-fitting CEFFE concentrations (2%) identified using the mice experiment based on the embryo development rates. However, the control group was the standard media. Oocytes were fertilized and cultured continuously from day 0 through day 5 or 6, with a daily medium renewal in both groups.

The quality of cleavage-stage embryos was evaluated using a standardised morphological scoring system based on blastomere number, fragmentation and symmetry [18]. A score of ≥7 for an embryo indicated good quality, and a score of ≥9 indicated top quality. The quality of blastocyst-stage embryos at day 5 or 6 was assessed using the Gardner grading system; [19] a blastocyst with a score ≥ 3 BC was considered as an available embryo, and that with a score ≥ BB as top-quality. The freeze-all strategy was performed; one or two cleavage embryos were frozen, and the remaining embryos continued in culture and were frozen at the blastocyst stage if they were suitable for vitrification.

### Examination of aneuploidy

Blastocyst biopsies were performed on days 5 or 6, and 5–8 trophectoderm (TE) cells were collected for each test. Whole-genome amplification and sequencing library construction of the biopsied samples were performed using the preimplantation genetic testing for aneuploidy kit (PGT-A, Basecare Medical, China). All sequencing data were aligned to reference sequences in the human genome (NCBI Build37/hg19), and copy number variations (CNVs) were screened using the circular binary segmentation algorithm. In this study, a copy number (CN) value of ≥2.7 or ≤1.3 indicated the presence of aneuploidy on autosomes or X chromosomes in females. A CN value of ≥1.7 or =0 indicated the presence of aneuploidy on sex chromosomes in males. Moreover, CNVs > 4 M and 30–70% mosaicism have been reported.

### Embryo warming and transfer

We randomly assigned 43 women to undergo day 3 or 5/6 thawing embryo transfer using the embryos developed in the CEFFE group ($n=22$) or control group (without CEFFE, $n=21$). The warming protocol was performed using traditional methods following the manufacturer's instructions of the Vit Kit (Kitazato Biopharma, Japan). We transferred one or two cleavage embryos or one blastocyst on days 5 or 6. We recorded serum beta-human chorionic gonadotropin levels, implantation rate, clinical pregnancy rate, miscarriage rate and live birth rate.

### Statistical analysis

Data were analysed and presented using SPSS software (version 23.0) and GraphPad Prism (version 8.0). Categorical data were evaluated using the chi-squared

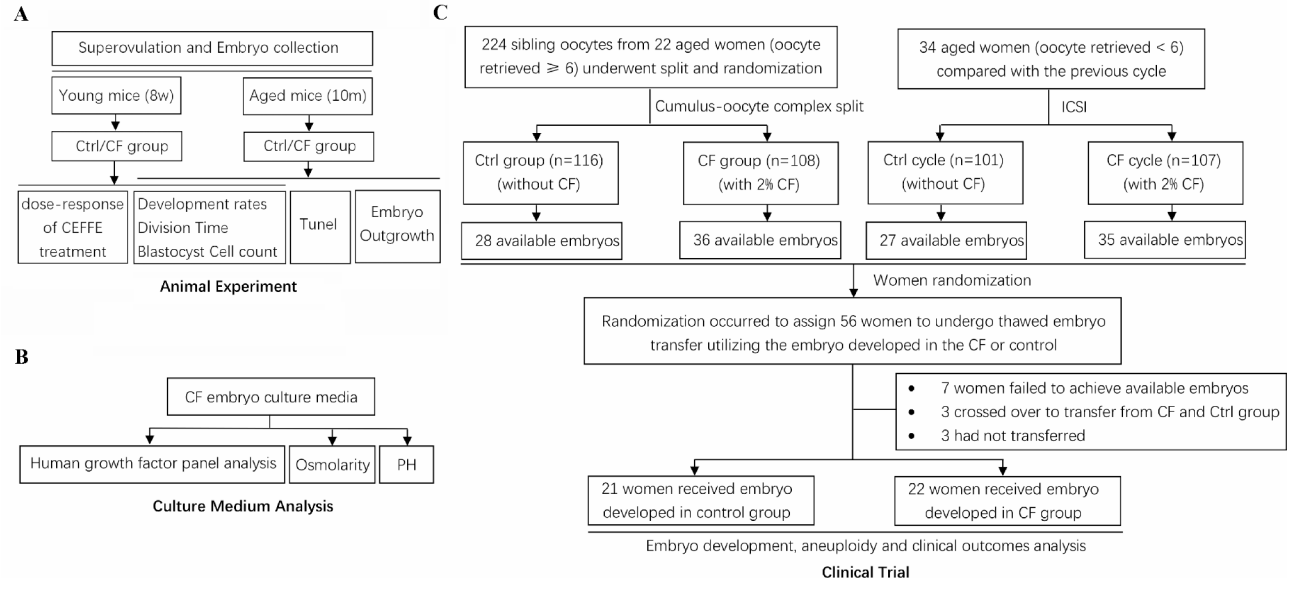


**Fig. 1** Experimental flowchart. **a**. Animal Experiment. CF, CEFFE. **b**. Culture Medium analysis. **c**. Clinical Trial. CEFFE, Cell-free fat extract

($\chi 2$) or Fisher's tests, and continuous data were analysed using the student's t-test. Data are expressed as the mean ± standard deviation, and $P < 0.05$ were considered statistically significant.

## Results

### CEFFE improves embryo cell division and blastocyst cell number of young mice

For the 8-week-old mice, treatment with different concentrations of CEFFE did not affect the percentage of embryos that developed into blastocysts. However, the percentage of hatched blastocysts increased in the 2% CEFFE treatment group (80.42% vs. 70.14%), but not significantly (Fig. 2a-b). Furthermore, we tracked the developmental stages in a timely manner using a time-lapse imaging system to identify the effects of CEFFE on embryonic cell division. Compared with embryos cultured in sequential media alone, the 2% CEFFE treatment resulted in considerably faster development times to 2-cell cleavage, which continued through the morula stage. However, no significant difference was observed between the 1% or 5% CEFFE groups and the control group (Fig. 2c). Moreover, immunofluorescence images obtained after the differential cell staining of blastocysts showed that supplementing the culture medium with 2% CEFFE resulted in a significant increase in blastocyst TE and ICM numbers, leading to an increase in the total cell numbers (Fig. 2d-e). These results indicated that 2% CEFFE promoted the development of embryos derived from young mice.

### CEFFE promotes embryo development and blastocyst cell number of aged mice

We collected the fertilized eggs from the ampulla of 10-month-old female mice, and recorded the process of in vitro embryo development to investigate further the effect of 2% CEFFE on aged mouse embryo cultures. We discovered that CEFFE had no obvious influence on the percentage of embryos that developed to the blastocyst stage but considerably increased the rate of hatched blastocysts (Fig. 3a-b). Besides, short-term CEFFE supplementation (after fertilization until the 8-cell stage) also significantly increased the rate of hatched blastocysts (55.26 vs. 68.80, $P < 0.05$) in aged mice. Moreover, CEFFE displayed significantly increased total blastocyst and TE cell numbers than the controls (Fig. 3d-e). No obvious differences in the embryo cell division time were observed in blastocysts derived from aged mice, regardless of CEFFE treatment (Fig. 3c).

### Embryos derived from aged mice reduce cell apoptosis and promote blastocyst adhesion with CEFFE supplementation

TUNEL analysis was performed in aged mice to measure embryo activity. CEFFE supplementation produced a significantly lower ratio of apoptotic cells compared with the control (Fig. 4). Hatched blastocysts obtained from the control or CEFFE group were co-cultured with HEEC layers to mimic embryo attachment and the invasion of the endometrial epithelium at implantation. The results showed that CEFFE supplementation improved embryo adhesion (Fig. 5a-b) and invasive capabilities (Fig. 5c).

### Growth factor content and time variation in growth factor concentrations in CEFFE supplementation

We maintained the 2% CEFFE supplementation medium for 5 d and analysed it using multiplex immunoassay to verify the underlying mechanism of CEFFE treatment. High levels of growth factors and cytokines identified in CEFEE samples by previous mass spectrometry technology were detected following CEFFE supplementation. The fresh 2% CEFFE supplementation mediums based on four CEFFE batches, include FGF-2 (127.11 ± 18.26 pg/ml), HGF (39.67 ± 5.33 pg/ml), EGF (2.55 ± 0.07 pg/ml), BDNF (2.12 ± 0.56 pg/ml), PDGF (1.85 ± 0.17 pg/ml), SCF (1.86 ± 0.29 pg/ml), LIF (1.14 ± 0.25 pg/ml) and PIGF-1 (0.95 ± 0.11 pg/ml). However, the growth factor concentrations decreased gradually (Additional Fig. 1). We renewed the mediums in the CEFFE and control groups to achieve the best effect on in vitro embryo culture. Embryos can develop normally with an osmolarity range of 250–300 mOsm/kg and a pH range of 7.2–7.4 in vitro. Our results showed that the 2% CEFFE-supplemented medium had a suitable osmolarity (289.50 ± 2.12 vs. 287.00 ± 3.46) and pH (7.39 ± 0.01 vs. 7.36 ± 0.01).

### Human embryos cultured with CEFFE formed normal karyotype

The preclinical safety experiment involved a PGT-A analysis of 30 blastocysts (control group, $n = 22$; CEFFE group, $n = 8$) from six parents, and the chromosome euploidy analyses suggested that the CEFFE enriched medium was the most compatible with no harmful effects (Additional Table 1 and Additional Fig. 2).

### CEFFE improved day 3 embryo quality in aged patients

We selected 22 aged patients with ≥6 oocytes retrieved using the "sibling oocyte split" approach. Notably, 116 cumulus-oocyte complexes were cultured in the control medium and 108 in the CEFFE-supplemented medium. Additional Table 2 shows the patients' baseline clinical characteristics. We compared embryo developmental outcomes between the control and CEFEE protocols during the same cycle for each patient (Table 1). Notably, both groups had similar fertilization and embryo cleavage rates. However, the rate of day 3 ≥ 8 blastomere number embryos (40.74% vs. 25.58%, $P = 0.037$) and high-quality embryos (33.33% vs. 19.51%, $P = 0.045$) in the CEFFE group was significantly higher than that in the control

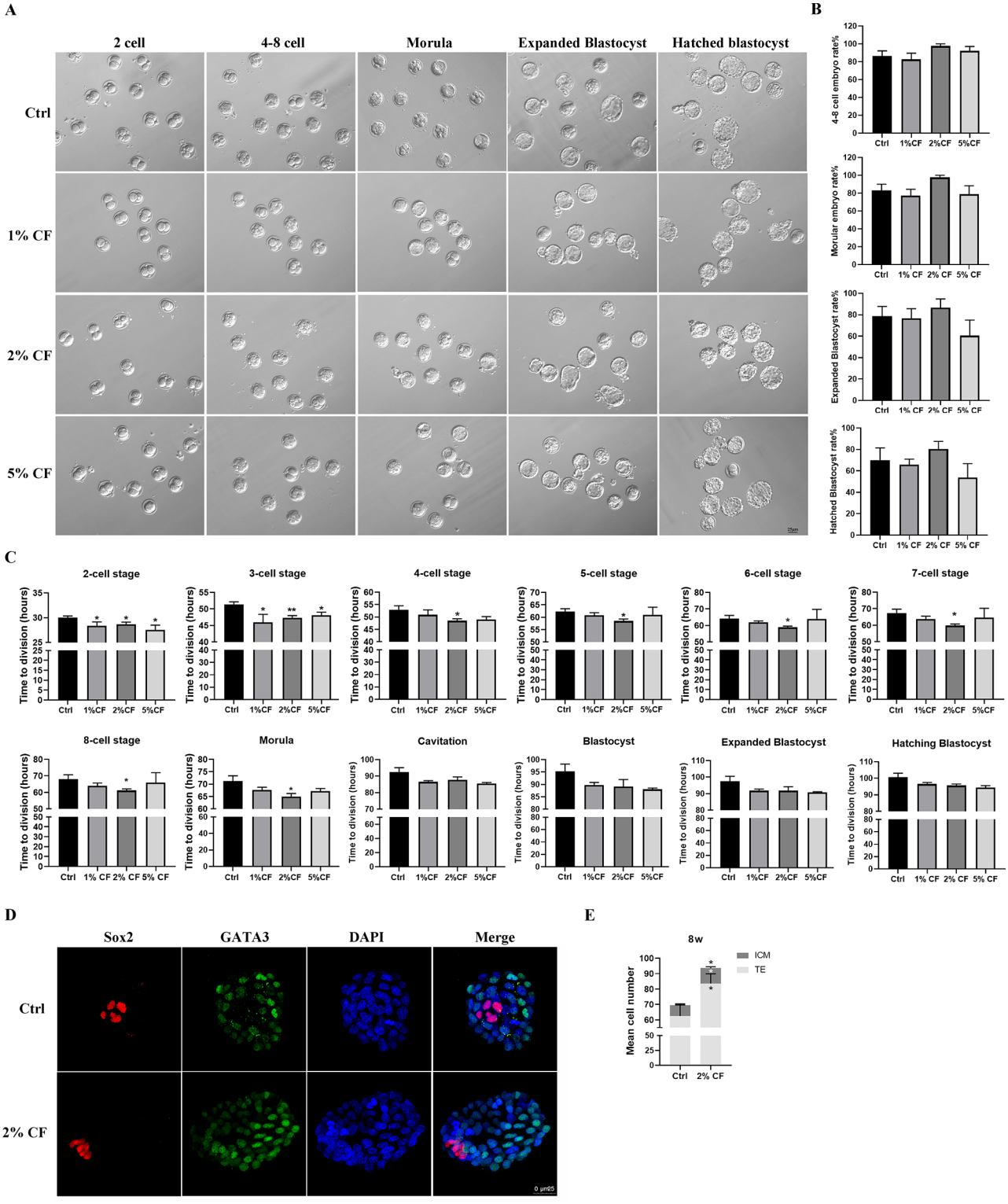


**Fig. 2** Impact of CEFFE supplementation on the development of embryos derived from young mice. **a-b**. Effect of different CEFFE concentrations on embryo development ($n=4$). **c**. Cell cleavage and development times (h) of 8-week-old mouse embryos cultured in IVF media supplemented with or without different CEFFE concentrations were measured in hours post hCG injection ($n=6$). **d-e**. Immunofluorescence staining and blastocyst cell count following culture in media supplemented with or without CEFFE ($n=6$). Significance was determined using a student's t-test, and data are presented as the mean ± SEM. *$P<0.05$; **$P<0.01$. CEFFE, Cell-free fat extract; IVF, invitro fertilization; hCG, human chorionic gonadotropin; SEM, standard error of the mean

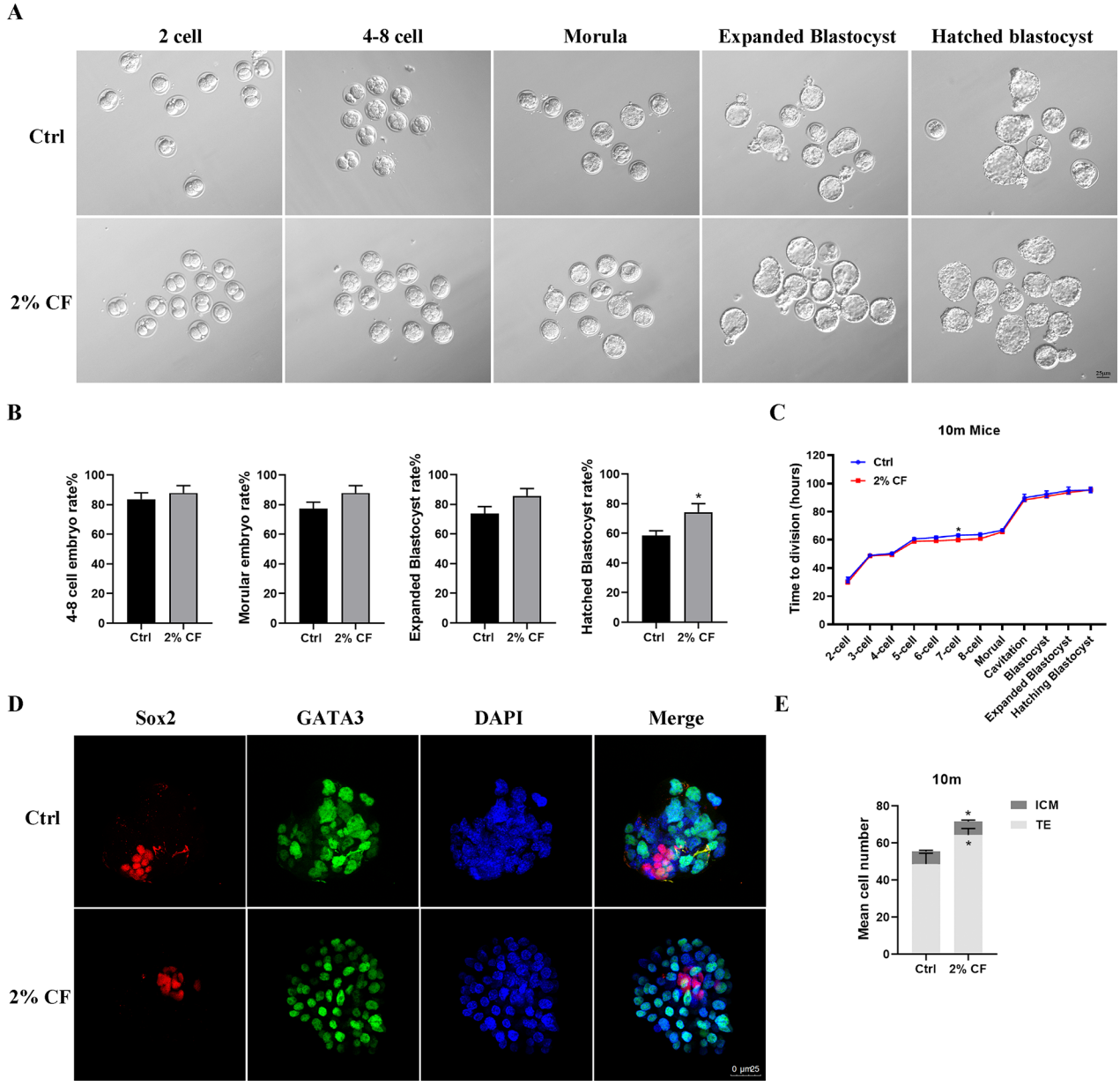


**Fig. 3** Impact of CEFFE supplementation on the development of embryos derived from aged mice. **a-b**. Adding 2% CEFFE to embryo culture media increased the rate of development to the hatched blastocyte stages ($n=8$). **c.** Cell cleavage and development times (h) of 10-month-old mouse embryos cultured in IVF media supplemented with or without 2% CEFFE ($n=8$). **d-e**. Immunofluorescence staining and blastocyst cell count following culture in media supplemented with or without 2% CEFFE ($n=8$). Significance was determined using a student's t-test, and data are presented as the mean ± SEM. *$P<0.05$. CEFFE, Cell-free fat extract; IVF, invitro fertilization; SEM, standard error of the mean

group. The rate of useable embryos (43.37% vs. 32.18%, $P=0.132$) was also increased, but not significantly.

We selected 34 aged patients with < 6 oocytes retrieved and 99 MII oocytes digested from 107 cumulus-oocyte complexes were subjected to ICSI and cultured in the CEFFE-supplemented medium. Additional Table 3 summarises the patients' demographic and baseline characteristics. The CEFFE intervention cycle had higher rates of day 3 available embryos (69.14% vs. 50.67%, $P=0.019$), day 3 ≥ 8 blastomere number embryo (44.44% vs. 28.00%, $P=0.033$) in the CEFFE intervention cycle than the previous cycle (Table 2).

### CEFFE improved pregnancy outcomes in aged patients

For the embryo transfer trial, the 43 women scheduled to receive thawed embryos were split into two groups: 22 in the CEFFE group and 21 in the control group. They received transfer embryos from the CEFFE-supplemented or the control medium, respectively. The two groups showed comparable baseline values (Additional

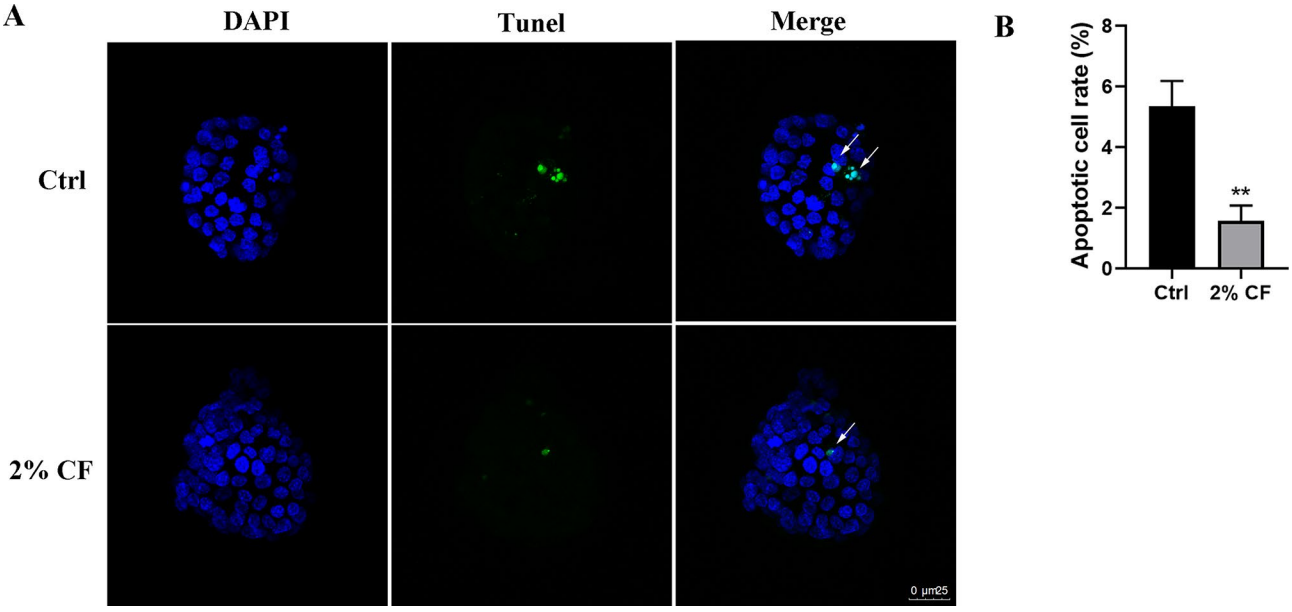


**Fig. 4** Effects of CEFFE supplementation on cell apoptosis. **a-b**. Representative images and apoptotic cell rate in blastocysts from control and CEFFE media ($n=8$). Scale bars = 25 μm. Significance was determined using a student's t-test, and data are presented as the mean ± SEM. $^{**}P<0.01$. CEFFE, Cell-free fat extract; SEM, standard error of the mean

Table 4). Embryos transferred from the CEFFE group showed an improved implantation rate (51.85% vs. 22.22%, $P=0.024$) and clinical pregnancy rate (59.09% vs. 28.57%, $P=0.044$) compared with those of the control group (Table 3). However, due to the small sample size, the rate of implantation and clinical pregnancy showed an increasing trend but not statistically significant in CEFFE group when stratified by the stage of the embryo. Also, the improvement using CEFFE was trending positively correlated with the rates of ongoing pregnancy/live births (40.91% vs. 19.05%, $P=0.119$). In addition, of the seven births in the CEFFE group, there were no neonatal complications, low birth weight babies, congenital anomalies, or neonatal death reports. There was only one case of preterm birth at 37 weeks due to maternal gestational diabetes and preeclampsia (Additional Table 5).

## Discussion

In this study, we assessed the effect of CEFFE supplementation on in vitro embryo culture using a naturally aging mouse model (mirroring women aged 35–40 years), and its efficacy in influencing embryo development and pregnancy rates in older women with IVF failure. CEFFE promoted embryo development and implantation in aged mice, probably by accelerating cell division, inhibiting cell apoptosis, and promoting blastocyst adhesion and invasion. CEFFE supplementation in human embryo culture media improved the embryological and clinical outcomes, particularly in older women with recurrent poor-quality embryo cycles.

Commercially available culture media lack cytokines. However, the absence of growth factors in the embryo culture medium may cause imbalances and stress in the embryos and slow their development [20, 21]. Previous studies have attempted to identify a superior embryo culture medium closer to the in vivo environment. Vascular endothelial growth factor (VEGF), derived maternally, promotes preimplantation embryonic development *in vitro.* [22] Medium enrichment of GM-CSF, HB-EGF, and LIF for human embryo culture improved live births without affecting birth outcomes [23]. Insulin supplementation of the embryo culture medium improves clinical pregnancy rates [4]. Previous proteomic spectrometry data [8] and our enzyme-linked immunosorbent assay results showed that CEFFE is rich in FGF-2, EGF, LIF, G-CSF, and VEGF, suggesting its potential as a potential growth factor integration medium, with a daily renewal. Our previous study also showed that CEFFE injection improved the number of retrieved oocytes, blastocyst formation rate, and litter size in aged mice and those with POI in vivo, with no side effects observed in parent mice and their pups [12, 13]. Therefore, we evaluated the effects of CEFFE on embryo cultures in vitro.

Notably, C57BL/6 mice embryos grow more slowly and are more sensitive to culture conditions because of lower production of autocrine trophic ligands *in vitro.* [24] Therefore, 2% CEFFE might be the best concentration for culturing C57BL/6 mice embryos. Adding 2% CEFFE to the culture media of young mouse preimplantation embryos accelerated their development from 2-cell cleavage to the morula stage and significantly improved

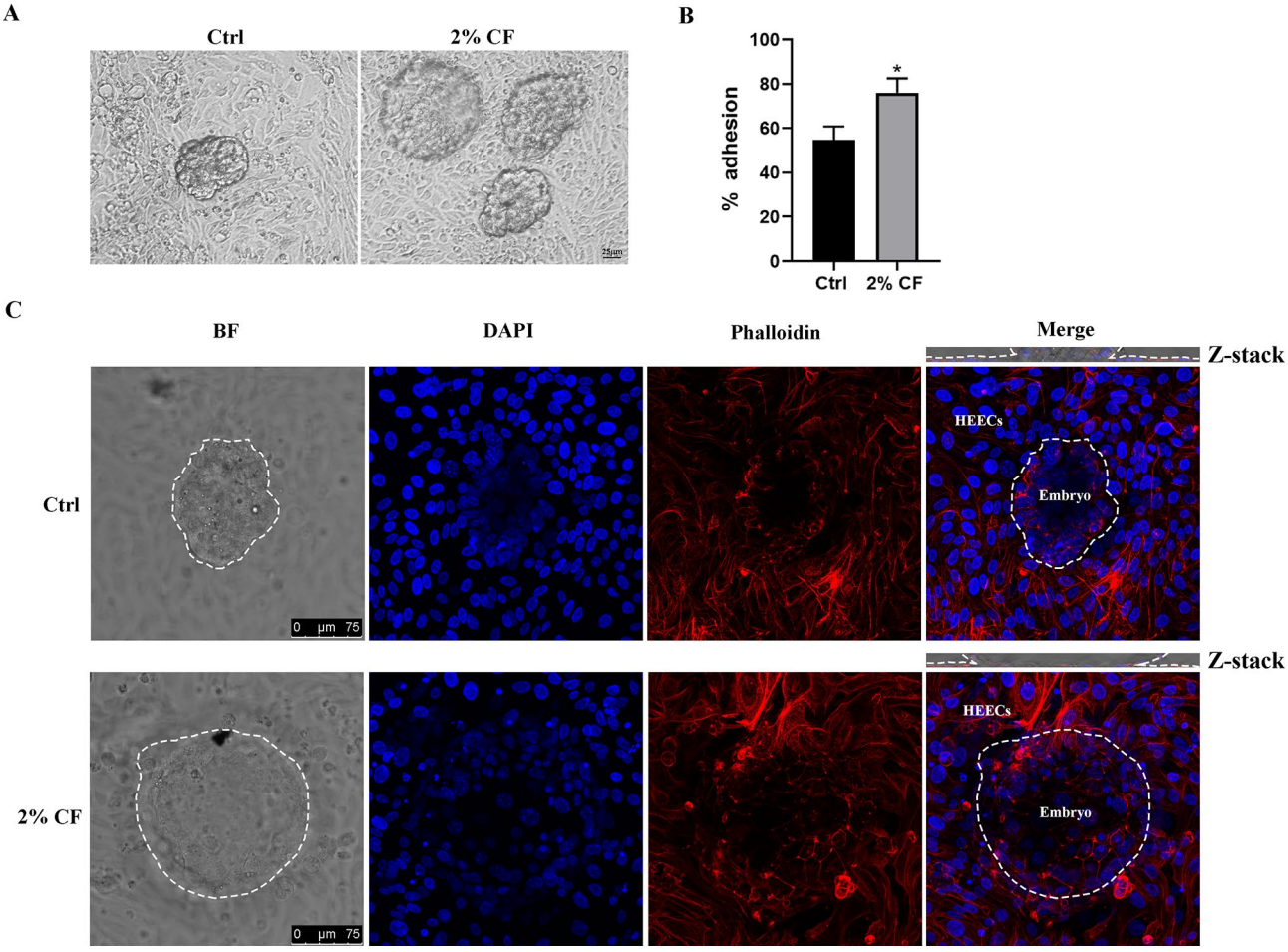


**Fig. 5** Effects of CEFFE supplementation on embryo adhesion and invasion. **a-b**. Representative images of adhesive mouse blastocysts and blastocyst adhesion rate between the control and CEFFE groups ($n = 5$). Scale bars = 25 µm. **c**. Co-cultures were fixed after 24 h and stained with phalloidin (red) and DAPI (blue). Fluorescence microscopy with optical sectioning allowed for determining embryonic breaching of the HEEC layer ($n = 5$). Dotted lines indicate the embryo-HEEC interface and Z-stacks of optical sections in upper panels. Scale bars = 75 µm. Significance was determined using a student's t-test, and data are presented as the mean ± SEM. *$P < 0.05$. CEFFE, Cell-free fat extract; HEEC, human endometrial epithelial cell; SEM, standard error of the mean

blastocyst cell numbers. However, we also found the developmental rates in the group with 5% CEFFE tended to decrease after compaction. Appropriate osmolarity and PH are required for embryonic development, as different pure CEFFE batches have osmolarity range from 280 to 320 mOsmol/kg and PH range from 6.90 to 7.10. Our results showed that 2% CEFFE-supplemented medium had a suitable osmolarity (289.50 ± 2.12 vs. 287.00 ± 3.46) and pH (7.39 ± 0.01 vs. 7.36 ± 0.01). We wonder if 5% CEFFE-supplemented medium has inappropriate osmolarity or PH, thus decreasing the developmental rates after compaction. During the in vitro culture of embryos, apoptosis can be induced by oxidative stress caused by ROS accumulation, leading to a lower blastocyst rate, low quality, and increased apoptotic nuclei [25–27]. Growth factors promote preimplantation embryonic growth by stimulating mitosis and cell proliferation and a decrease in apoptosis [28]. CEFFE contains transforming growth factor (TGF)β, VEGF, EGF and other growth factors, and exerts anti-oxidant, anti-apoptotic, and proliferative effects in osteocytes and Achilles tendon, endometrial, and ovarian granulosa cells [10, 12, 14]. Previous studies show that CEFFE activates the TGFβ pathway and might promote cell proliferation and antagonise apoptosis of ovary granulosa cells. CEFFE promotes the proliferation of human dermal papilla cells by inhibiting cell cycle arrest and apoptosis [12, 29]. Compared with the control group, the CEFFE medium significantly improved blastocyst cell numbers and hatched blastocyst development and reduced apoptosis frequency in aged mice. Our in vitro implantation model also suggests that CEFFE supplementation is advantageous for TE function regarding attachment and the invasion of endometrial epithelial cells. Therefore, our results show that adding CEFFE to the medium promotes embryonic development and implantation in aged mice.

Studies investigating growth factor supplementation in animal models have revealed improved blastocyst

**Table 1** Embryologic characteristics in the trial groups (sibling-oocyte-split)

| Embryological outcomes | Control group (*n* = 22) | CEFFE group (*n* = 22) | *P* value |
|---|---|---|---|
| No. of cumulus-oocyte complexes split | 5.27 ± 2.53 | 4.91 ± 1.93 | 0.594 |
| Fertilization rate (2PN) of ICSI[a] | 80.26% (61/76) | 82.09% (55/67) | 0.781 |
| Fertilization rate (2PN) of IVF[b] | 73.33% (22/30) | 75.00% (27/36) | 0.877 |
| Cleavage rate | 98.85% (86/87) | 97.59% (81/83) | 0.533 |
| Day3 available embryo rate[c] | 59.30% (51/86) | 61.73% (50/81) | 0.749 |
| Day 3 ≥ 8 BL embryo rate[d] | 25.58% (22/86) | 40.74% (33/81) | 0.037* |
| Day 3 good-quality embryo rate[e] | 19.51% (16/82) | 33.33% (27/81) | 0.045* |
| Blastocyst formation rate | 36.00% (27/75) | 41.27% (26/63) | 0.526 |
| Available blastocyst rate | 62.96% (17/27) | 69.23% (18/26) | 0.630 |
| Rate of useable embryos per fertilized oocyte | 32.18% (28/87) | 43.37% (36/83) | 0.132 |

[a]Fertilization rate (2PN) of ICSI (%) = No. of 2PN zygotes/No. of microinjected MII oocytes

[b]Fertilization rate (2PN) of IVF (%) = No. of 2PN zygotes/No. of inseminated oocytes

[c]Day 3 available embryo rate = No. of day 3 available embryos/No. of cleavage embryos

[d]Day 3 ≥ 8 BL embryo rate = No. of day 3 ≥ 8 blastomere number (BL) embryos / No. of cleavage embryos

[e]Day 3 good-quality embryo rate = No. of day 3 good quality embryos/No. of 2 PN cleavage embryos

Data are expressed as mean ± SD or frequency. The difference between the control and CEFFE was analysed by Pearson Chi-Square Continuity Correction, except "No. of cumulus-oocyte complexes split", which was calculated by independent samples t-test. *$P < 0.05$

**Table 2** Embryologic characteristics in the trial groups (comparison with previous cycle)

| Embryological outcomes | Control cycle | CEFFE cycle | *P* value |
|---|---|---|---|
| No. of oocytes retrieved | 2.97 ± 2.56 | 3.15 ± 2.27 | 0.765 |
| Maturation rate | 92.08% (93/101) | 92.52% (99/107) | 0.904 |
| Fertilization rate (2PN) of ICSI | 78.49% (73/93) | 80.81% (80/99) | 0.690 |
| Cleavage rate | 100.00% (75/75) | 100.00% (81/81) | / |
| Day3 available embryo rate | 50.67% (38/75) | 69.14% (56/81) | 0.019* |
| Day 3 ≥ 8 BL embryo rate | 28.00% (21/75) | 44.44% (36/81) | 0.033* |
| Day 3 good-quality embryos rate | 27.40% (20/73) | 37.50% (30/80) | 0.183 |
| Blastocyst formation rate | 24.14% (14/58) | 33.87% (21/62) | 0.241 |
| Available blastocyst rate | 71.43% (10/14) | 76.19% (16/21) | 0.752 |
| Rate of useable embryos per fertilized oocyte | 36.00% (27/75) | 43.21% (35/81) | 0.358 |

Data are expressed as mean ± SD or frequency. The difference between the control and CEFFE was analysed by independent samples t-test or Pearson Chi-Square Continuity Correction. *$P < 0.05$

**Table 3** Clinical outcomes by trial group

| Outcome | Control (*n* = 21) | CEFFE (*n* = 22) | *P* |
|---|---|---|---|
| Number of embryos per transfer | 1.29 ± 0.46 | 1.23 ± 0.43 | 0.670 |
| Proportion of day 3 embryos | 37.04% (10/27) | 40.74% (11/27) | 0.780 |
| Proportion of blastocysts | 62.96% (17/27) | 59.26% (16/27) | 0.780 |
| Proportion of good quality embryos | 29.63% (8/27) | 33.33% (9/27) | 0.770 |
| Biochemical pregnancy (total) | 66.66% (14/21) | 68.18% (15/22) | 0.916 |
| Biochemical pregnancy (day 3 embryos) | 57.14% (4/7) | 62.50% (5/8) | 0.833 |
| Biochemical pregnancy (blastocysts) | 71.43% (10/14) | 71.43% (10/14) | 1.000 |
| Implantation rate (total) | 22.22% (6/27) | 51.85% (14/27) | 0.024* |
| Implantation rate (day 3 embryos) | 20.00% (2/10) | 54.55% (6/11) | 0.104 |
| Implantation rate (blastocysts) | 23.53% (4/17) | 50.00% (8/16) | 0.114 |
| Clinical pregnancy (total) | 28.57% (6/21) | 59.09% (13/22) | 0.044* |
| Clinical pregnancy (day 3 embryos) | 28.57% (2/7) | 62.50% (5/8) | 0.189 |
| Clinical pregnancy (blastocysts) | 28.57% (4/14) | 57.14% (8/14) | 0.127 |
| Ongoing pregnancy/Live birth (total) | 19.05% (4/21) | 40.91% (9/22) | 0.119 |
| Ongoing pregnancy/Live birth (day 3 embryos) | 14.29% (1/7) | 37.50% (3/8) | 0.310 |
| Ongoing pregnancy/Live birth (blastocysts) | 21.43% (3/14) | 42.86% (6/14) | 0.225 |

Data are expressed as frequency. The difference between the control and CEFFE was analysed using Pearson Chi-Square Continuity Correction. *$P < 0.05$

development rates, implantation, and birth rates. However, research on growth factor supplementation in human culture media is limited [30]. Therefore, we assessed the effects of CEFFE supplementation in human embryo culture. An increased proportion of high-quality day 3 embryos is suggestive of blastocyst formation, implantation, and pregnancy [31]. We observed a considerably higher proportion of ≥ 8 BL and high-quality cleavage-stage embryos in the CEFFE-supplemented medium, which preliminarily supports the beneficial effects of CEFFE in human culture medium. Cytokine enrichment likely reduces biochemical stress, maintains embryo plasticity, stimulates hatching proteases, and saves energy for post-implantation development. Notably, several studies have shown an association between infertility and recurrent pregnancy loss with the dysregulation of growth factors and cytokines [5, 32, 33]. Failed IVF-ET cycles in aged patients may result from the failure of the embryo to hatch for implantation [23, 34]. In this study, adding CEFFE improved the implantation rate and clinical pregnancy rate, suggesting its effectiveness in addressing possible defects in hatching or implantation to improve clinical outcomes in aged patients. Moreover,

the chromosome euploidy analyses and the follow-up of newborns suggested that CEFFE supplementation was the most compatible with safety.

Our previous study demonstrated that CEFFE contained 1767 active proteins. Of these, 131 and 141 proteins are associated with cell proliferation and apoptosis, respectively [12]. Moreover, 'Focal adhesion' (56 proteins), 'Adherens junction' (22 proteins) 'Regulation of actin cytoskeleton' (57 proteins) and 'Tight junction' (43 proteins) items were enriched in Kyoto encyclopaedia of genes and genomes enrichment analysis of CEFFE (sTable 6). We speculated that those proteins possibly contribute to pro-proliferation, pro-adhesion, and pro-invasion, which are crucial to improving embryo development and implantation. However, the components which play a crucial role in CEFFE supplementation are still unknown. The functional fraction of CEFFE needs to be dissected in future.

This study has several strengths. The effectiveness and safety of CEFFE were demonstrated in vivo and in vitro using a naturally ageing mouse model. This clinical trial examined aneuploidy, assessed embryo quality, and explored live births. In addition, the sibling-oocyte-split model served as a variable to evaluate participants' embryo development in vitro, allowing for a between-group evaluation of clinical outcomes.

This study has some limitations. It did not investigate the epigenetic or transcriptomic information between a developing embryo and its surrounding microenvironment, as well as the multi-faceted interactions within the factors of CEFFE. Besides, the stage-specific temporal treatment of CEFFE might be needed to unearth the most efficient way of CEFFE treatment in further study.

## Conclusion

We demonstrated that CEFFE supplementation of the human embryo medium with abundant growth factors effectively improved clinical outcomes with no adverse consequences. However, large-scale trials and long-term tracking of CEFFE in IVF babies are warranted before CEFFE can be used in personalised care programs.

### Abbreviations

| | |
|---|---|
| BDNF | Brain-derived neutrophilic factor |
| bFGF | Basic fibroblast growth factor |
| CEFFE | Cell-free fat extract |
| CN | Copy number |
| CNVs | Copy number variations |
| DAPI | 4′, 6-Diamidino-2-Phenylindole |
| EGF | Epidermal growth factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HB-EGF | Heparin-binding epidermal growth factor-like growth factor |
| HEECs | Human endometrial epithelial cells |
| ICM | Inner cell mass |
| IGF1 | Insulin-like growth factor 1 |
| ICSI | Intracytoplasmic sperm injection |
| IVF | In vitro fertilization |
| LIF | Leukemia inhibitory factor |
| MII | Metaphase II |
| PBS | Phosphate-buffered saline |
| PGT-A | Preimplantation genetic testing for aneuploidy |
| POI | Primary ovarian insufficiency |
| ROS | Reactive oxygen species |
| TE | Trophoblast |
| TUNNEL | Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12958-024-01341-4.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

### Author contributions

XZ, YC, and AZ conceived of and designed the experiments. Clinical samples collected: HX, SZ, LX. XZ, HZ and JD performed experiments. XZ and XW analyzed the data. XZ and BX wrote the manuscript.

### Funding

This study was funded by the National Natural Science Foundation of China (grant numbers 82101800 and 82271730), the Shanghai Sailing Program (grant number 21YF1426600) and the National Key R&D Program of China (No. 2022YFC2703800).

### Data availability

No datasets were generated or analysed during the current study.

## Declarations

### Ethics approval and consent to participate

This study is conducted in accordance with the Declaration of Helsinki with ethical approval from the Ruijin Hospital Institutional Ethics Committee (number: 2022 – 216). Participants provided written informed consent before participation.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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Received: 8 August 2024 / Accepted: 23 December 2024
Published online: 17 January 2025

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## Publisher's note