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ABOUT COVER

Editorial Board Member of *World Journal of Stem Cells*, Dr. Alessandra Pelagalli is a Senior Researcher of veterinary physiology in the Department of Advanced Biomedical Sciences at the University of Naples. Having completed her Pharmacy Degree from the University of Naples in 1991, Dr. Pelagalli continued her postgraduate training and received her PhD in 1996. She became a Young Researcher at the University of Naples in 1999, working in animal platelet physiology and biochemistry. Her current research interests and publications focus on the roles and behavior of bone marrow mesenchymal stem cells in the differentiation processes after stimulation, water channel proteins in cell migration, and aquaporins in various tissues, such as gut and the male reproductive tract. (L-Editor: Filipodia)

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Basic Study

Application of cattle placental stem cells for treating ovarian follicular cyst

Shao-Yu Peng, Tsung-Hsin Wu, Tzu-Yi Lin, Ling-Yien Hii, Kok-Seong Chan, Tzu-Yen Fu, Shen-Chang Chang, Perng-Chih Shen, Kang-You Liu, Steven W. Shaw

ORCID number: Shao-Yu Peng 0000-0002-0934-4761; Tsung-Hsin Wu 0000-0002-3594-6950; Tzu-Yi Lin 0000-0002-5181-5571; Ling-Yien Hii 0000-0001-7155-4180; Kok-Seong Chan 0000-0002-7695-1465; Tzu-Yen Fu 0000-0001-6128-7592; Shen-Chang Chang 0000-0003-1746-9481; Perng-Chih Shen 0000-0002-9901-5274; Kang-You Liu 0000-0002-1637-781X; Steven W Shaw 0000-0002-3931-7297.

Author contributions: Peng SY and Shaw SW designed and coordinated the study; Peng SY, Wu TH, and Fu TY performed the experiments; Chang SC and Shen PC analyzed the data; Peng SY, Liu KY, and Shaw SW interpreted the data; Peng SY drafted the manuscript; Lin TY, Hii LY, and Chan KS revised the manuscript; Shaw SW critically reviewed the manuscript; All authors were involved in the amendment and approval of the final manuscript.

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Shao-Yu Peng, Tsung-Hsin Wu, Tzu-Yen Fu, Perng-Chih Shen, Kang-You Liu, Department of Animal Science, National Pingtung University of Science and Technology, Pingtung 912, Taiwan

Tzu-Yi Lin, Steven W. Shaw, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan

Ling-Yien Hii, Kok-Seong Chan, Steven W. Shaw, Department of Obstetrics and Gynecology, Taipei Chang Gung Memorial Hospital, Taipei 105, Taiwan

Shen-Chang Chang, Kaohsiung Animal Propagation Station, Livestock Research Institute, Council of Agriculture, Executive Yuan, Pingtung 912, Taiwan

Steven W. Shaw, Prenatal Cell and Gene Therapy Group, Institute for Women's Health, University College London, London WC1E 6HU, United Kingdom

Corresponding author: Steven W. Shaw, MD, PhD, Associate Professor, Director, Department of Obstetrics and Gynecology, Taipei Chang Gung Memorial Hospital, No. 199 Dunhua North Road, Taipei 105, Taiwan. dr.shaw@mc.com

Abstract

BACKGROUND

High humidity and temperature in Taiwan have significant effects on the reproductivity of Holstein cattle, resulting in the occurrence of bovine ovarian follicular cyst (OFC). Because of economic loss from OFC, manual rupture and hormone injection have been advocated for the management of OFC. However, these incomplete treatments increase hormone resistance in cattle. Mesenchymal stem cells (MSCs) derived from placental stem cells (PSCs) demonstrate potential properties for the treatment of several diseases *via* promoting angiogenesis and immune modulation.

AIM

To establish the possibility of cattle placental stem cells (CPSCs) as a treatment modality for OFC of cows in Taiwan.

METHODS

The cows with OFC were divided into three groups: control (BC1 and BC2),

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hormone (H1 and H2), and CPSC (PS1 and PS2) treatment groups. In the hormone treatment group, the cows were given gonadotrophin-releasing hormone (GnRH)-prostaglandin-GnRH intramuscular injection with or without drainage of follicular fluid. In the CPSC treatment group, CPSCs were isolated from the placenta after labor. With the identification of surface antigen on stem cells, the cows were administered ovarian injection of 1×10^6 or 6×10^6 CPSCs with drainage. In all groups, OFC was scanned by ultrasound once a week for a total of seven times. The concentrations of estradiol and progesterone in serum were tested in the same period. The estrus cycle was analyzed by food intake and activity. If estrus was detected, artificial insemination was conducted. Then the cow was monitored by ultrasound for confirmation of pregnancy.

RESULTS

After 7 d of culture, CPSCs were successfully isolated from placental pieces. CPSCs significantly proliferated every 24 h and had high expression of MSC markers such as cluster of differentiation 44, as determined by flow cytometry. Ultrasound showed lower numbers of OFCs with drainage of follicular fluid. We achieved recovery rates of 0%, 50%, 50%, 75%, 75% and 75% in BC1, BC2, H1, H2, PS1, and PS2, respectively. Higher concentrations of progesterone were detected in the CPSC treatment groups. However, both hormone and CPSC treatment groups had no significant difference in the concentration of estradiol. The estrus rate was 0%, 100%, 25%, 75%, 75% and 75% in BC1, BC2, H1, H2, PS1, and PS2, respectively. The two fetuses were born in H2 and PS1. In brief, cows with CPSC injection achieved higher recovery, estrus, and inseminated conception rates.

CONCLUSION

CPSCs have efficacy in treating cows with OFC, and thus, may serve as an alternative treatment for reproductive disorders.

Key Words: Cow; Estrus rate; Ovarian follicular cyst; Placenta; Recovery rate; Stem cell

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Core Tip: Ovarian follicular cysts (OFCs) harm the reproductivity and milk production of cows. To deal with economic loss, this study established the possibility of using cattle placental stem cells (CPSCs) for treating OFC. We drained the follicular fluid and injected CPSCs into the ovaries. Then the CPSCs significantly proliferated and expressed high levels of cluster of differentiation 44. Elevated concentrations of progesterone in serum were observed. Decreased numbers of OFCs were shown by ultrasound. Cows with CPSC injection had higher recovery, estrus, and inseminated conception rates. These outcomes indicate the therapeutic potential of CPSCs for OFCs in cattle.

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INTRODUCTION

Taiwan is located in subtropical and tropical areas with high humidity and temperature. Such heat stress seriously affects the physiological and reproductive function of Holstein, including milk yield and conception^[1]. To produce more milk, cows should take in more nutrition. However, decreased food intake due to heat stress leads to poor reproductive function^[2,3]. This reproductive disorder represents the main economic loss in Taiwan.

Ovarian follicular cyst (OFC) is a common reproductive disorder caused by an imbalance in hormones from the hypothalamus and pituitary gland. This abnormal

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condition causes ovulation failure and decreases the profits for farmers^[4-6]. To solve this problem, manual rupture of ovarian follicles is used for treating OFC. However, this method decreases the conception rate if hemorrhage in ovaries occurs^[7]. Consequently, an alternative approach is using hormones, such as human chorionic gonadotrophin^[8] and gonadotrophin-releasing hormone (GnRH)^[7,9]. Unfortunately, this treatment increases hormone resistance or has no effect on cattle^[7-9].

Recently, the use of stem cells to repair or replace damaged tissue has been extensively studied^[10]. Stem cells secrete growth factors and anti-inflammatory factors^[11] and reduce fibrosis^[12]. Several studies have reported the success of stem cells for treating retinal^[13], liver^[14], and cardiac diseases^[15]. On the other hand, the placenta can be non-invasively collected as great sources of stem cells without much ethical concern^[16]. Many placental stem cells (PSCs) can be identified by the expression of mesenchymal stem cells (MSCs) and pluripotency markers^[17]. These cells can be differentiated into mesodermal-related cells^[18]. In addition, PSCs have therapeutic properties for the management of several diseases such as wound healing^[19], diabetes^[20], lung fibrosis^[21], among others^[22,23]. To date, the effect of cattle placental stem cells (CPSCs) on treating OFC is still under research.

The objective of this study was to establish the possibility of using CPSCs for the treatment of cattle with OFCs in Taiwan.

MATERIALS AND METHODS

Animal source

The placental tissues were collected from 3- to 4-year-old laboring cows of Holstein species from the National Pingtung University of Science and Technology. Holstein cows from three different pastures were all fed a total mixed ration composed of alfalfa, bermuda, sweet oats, and silage corn in free-stall barns. The OFCs were diagnosed by follicle size (> 25 mm), existence (> 10 d), and thickness of the follicular wall (< 3 mm).

Establishment and characterization of bovine PSCs

Isolation of stem cells: After labor, the placenta was collected and disinfected by 75% alcohol. Then the cleaned placenta was cut into small pieces and cultured in medium (α MEM containing 10% fetal bovine serum, M0894; Sigma, St. Louis, MO, United States and 1% penicillin/streptococcus, 10437 and 15140; Gibco, Mexico and the United States, respectively) in a 38.5 °C, 5% CO₂ incubator (Thermo, Electron Corporation, Waltham, MA, United States) for 7 d to isolate the cells.

Analysis of stem cell proliferation: After the sixth passage, CPSCs were suspended in 0.25% trypsin-EDTA (25200072; Gibco, Vancouver, Canada). Then 2×10^4 cells were plated into 6-well dishes. The cell number was measured with trypan blue staining at 12, 24, 48, 72, and 96 h.

Analysis of stem cell surface antigen: After calculating the number of CPSCs, 2.5×10^5 cells were centrifuged, followed by staining with the cell surface antigens cluster of differentiation 4 (CD4, MCA 1653F; Bio-Rad, Hercules, CA, United States), CD44 (MCA 2433F; Bio-Rad), and CD105 (MA5-11854; Thermo Fisher Scientific) for 30 min. The antibodies were removed by centrifugation at $270 \times g$ for 5 min. Finally, the stained cells were analyzed by flow cytometry (BD FACS Aria II; BD Biosciences, Franklin Lakes, NJ, United States).

Bovine PSC therapy for OCTs

CPSC therapeutic potential: The study design is shown in [Figure 1](#). A total of 19 cows with OFC were divided into three groups: control, hormone, and CPSC treatment groups. The BC1 control group was not treated. The BC2 control group was treated with follicular fluid drainage and saline injection. The H1 hormone treatment group was given intramuscular injection of GnRH (250 μ g)-prostaglandin (PG) (500 μ g)-GnRH (250 μ g) without drainage of follicular fluid. The H2 hormone treatment group was given intramuscular injection of GnRH-PG-GnRH with drainage of follicular fluid. The PS1 CPSC treatment group was given ovarian injection of 1×10^6 CPSCs with follicular fluid drainage. The PS2 CPSC treatment group was given ovarian injection of 6×10^6 CPSCs ([Figure 1](#)) with follicular fluid drainage. All of the suspended CPSCs were stored in Dulbecco's phosphate-buffered saline (21300; Gibco, United States) at 4 °C before use.

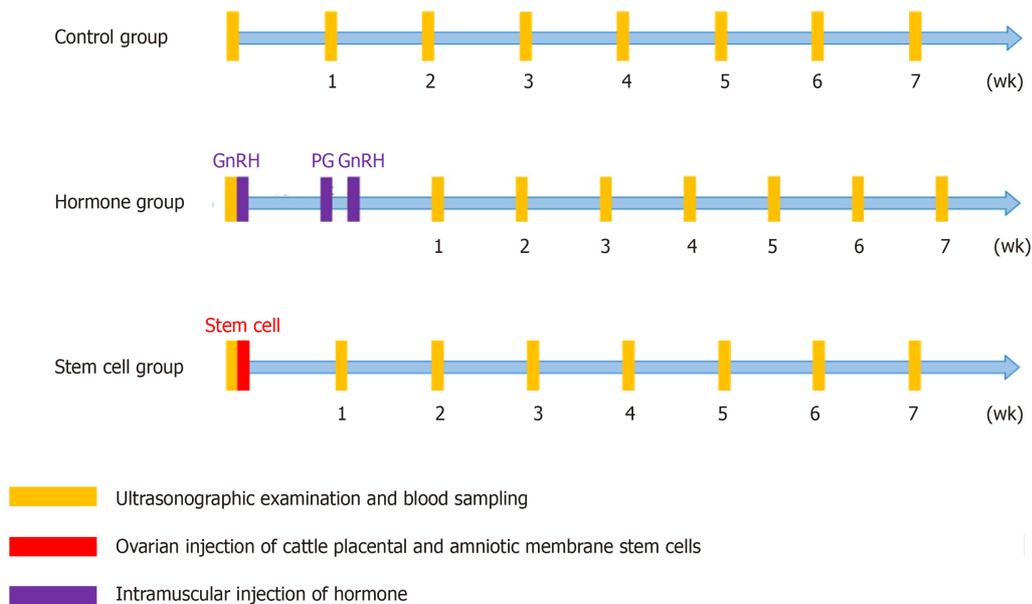


Figure 1 Design of the experiment. The serum of all cows with ovarian follicular cyst was collected to monitor the concentration of hormone every week for a total of seven times. In the hormone treatment group, the injection of prostaglandin (PG) was implemented 7 d after primary injection of gonadotropin-releasing hormone (GnRH). At 2 d after the injection of PG, secondary injection of GnRH was implemented.

Observation of ovarian follicle, estrus, and pregnancy status: The status of ovarian follicles was observed by the B-mode ultrasound scanner (iScan Draminski B-Mode) after treatment. The estrus detection system was used to determine the estrus cycle by analyzing feed intake and activity. When estrus was detected, artificial insemination was done after 8 to 12 h. Then ultrasound assessment was performed to confirm pregnancy.

Analysis of the hormone in serum: A total of 10 mL blood was collected in an anticoagulation-EDTA tube (367525; BD Vacutainer, Becton Dickinson, London, United Kingdom) from the tail veins of each experimental group, and stored at 4 °C for 30 min. The blood was centrifuged at 3000 rpm for 10 min to collect the serum. Then the serum was stored at -80 °C. Chemiluminescence was utilized to measure the concentrations of estradiol and progesterone in the serum (Centaur Immunoassay System; SIEMENS, Munich, Germany).

Statistical analysis

The data were analyzed with a statistical analysis system (SAS 9.4). A general linear model and Duncan's New Multiple Range Test were generated with statistical significance if $P < 0.05$.

RESULTS

After 3 to 7 d of culture, the spindle-shaped cells were isolated from placental pieces (Figure 2). There was a higher possibility of isolating the cells from the smaller placental pieces. The CPSCs calculated every 24 h in the sixth passage showed strong proliferation potential (Figure 3). The CPSCs expressed CD44 and slightly expressed CD4 and CD105 (Figure 4).

To the best of our knowledge, the status of the ovary varies in the estrus cycle with a change in hormones, such as progesterone (P4) and estradiol (E2). A high concentration of E2 and P4 confirmed the pregnancy, but cows with OFC had a low concentration of P4 (Figure 5). Both the hormone and CPSC treatment groups had no significant difference in E2 concentration, which may originate from variation in individual bovines (Figure 6A). Interestingly, the concentration of P4 increased in the hormone and CPSC treatment groups, showing the therapeutic potential for both treatments (Figure 6B).

Without treatment, the reproductive function of cows with OFC does not recover.

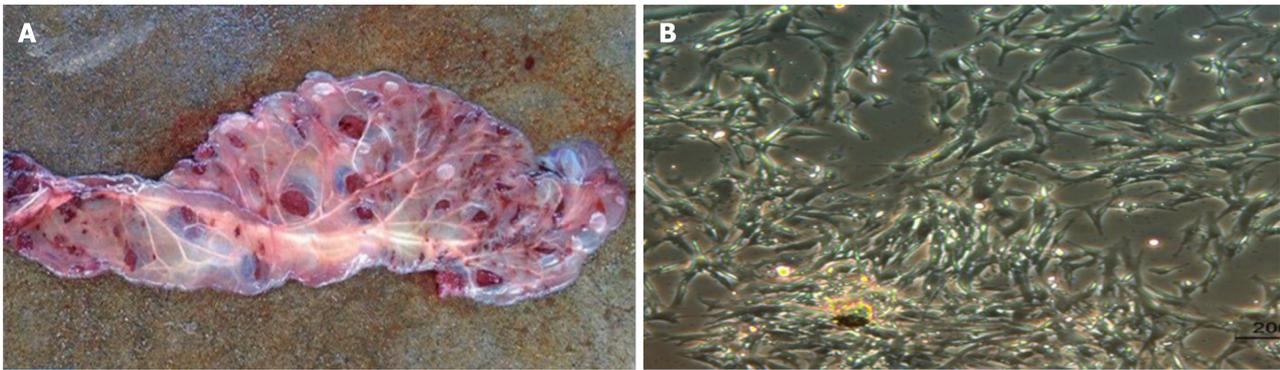


Figure 2 Isolation of cattle placental stem cells from placenta. A: The placenta collected from Holstein cow; B: The stem cell derived from bovine placenta after 7 d of culture. The uneven distribution of cattle placental stem cells led to different growth rates in each part of the dish.

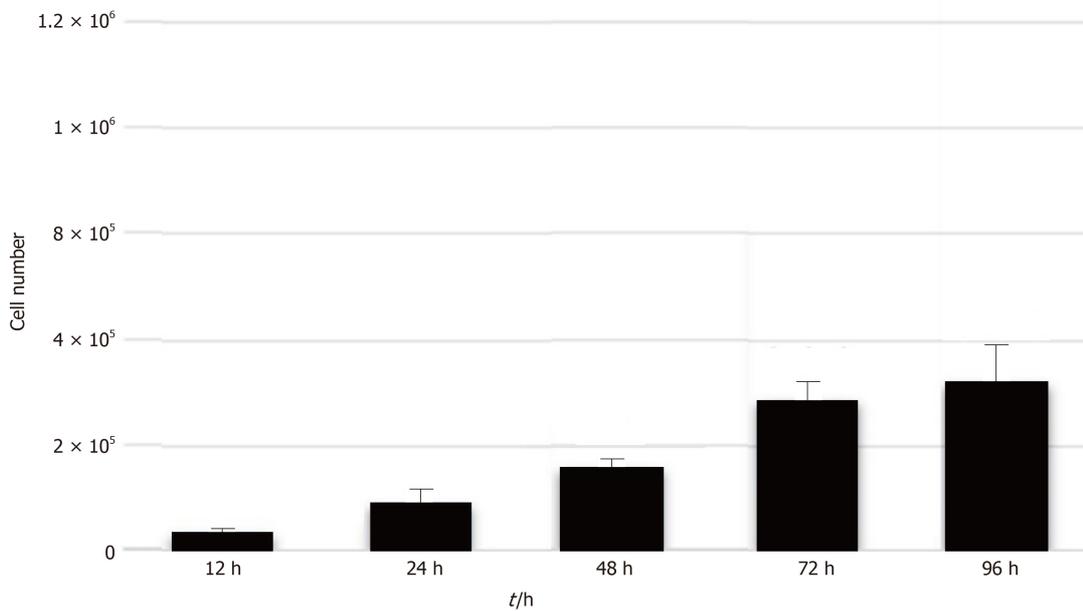


Figure 3 The proliferation of cattle placental stem cells. The number of cattle placental stem cells increased significantly from 12 h to 72 h. ^a*P* < 0.05; ^b*P* < 0.01; ^c*P* < 0.005.

The follicular cyst causes excessively sexual excitement. Then, artificial insemination might fail. These non-lactating cows cause significant economic loss for farmers. In our study, OFC subsided with artificial drainage of ovarian follicular fluid, but recurrence is probable (Figure 7). Therefore, treatment for OFC is needed. Hormone and CPSC injections were effective in managing OFC, as we achieved recovery rates of 50%, 75%, 75%, and 75% in H1, H2, PS1, and PS2, respectively (Table 1). Furthermore, the estrus rate was 100%, 25%, 75%, 75% and 75% in BC2, H1, H2, PS1, and PS2, respectively. Both H2 and PS1 demonstrated a 25% inseminated conception rate and gave birth to a heifer (Figure 8).

DISCUSSION

Placenta originating from the trophoblast forms chorionic villi, which allow healthy development of the embryo and fetus^[24]. Generally, the placenta is useless after delivery. Some researchers have begun using placenta as source of stem cells to improve the treatment of intractable disease^[17,18]. Our research showed that CPSCs had strong properties of proliferation and expression of MSC markers, similar to previous studies^[25,26]. CD44 secreted by MSCs is associated with cell homing and immunoregulation. Therefore, CD44 is able to reduce the inflammatory process in damaged

Table 1 Recovery rate, estrus detection rate, and inseminated conception rate of cows in each group within week 7

Group	Number of cows	Recovery rate, %	Estrus rate, %	Inseminated conception rate, %
BC1	1	0 (0/1)	0 (0/1)	0 (0/1)
BC2	2	50 (1/2)	100 (2/2)	0 (0/2)
H1	4	50 (2/4)	25 (1/4)	0 (0/4)
H2	4	75 (3/4)	75 (3/4)	25 (1/4)
PS1	4	75 (3/4)	75 (3/4)	25 (1/4)
PS2	4	75 (3/4)	75 (3/4)	0 (0/4)

BC1: Control group 1 (Without any treatment); BC2: Control group 2 (saline injection with follicular fluid drainage); H1: Hormone group 1 (GnRH-PG-GnRH intramuscular injection without follicular fluid drainage); H2: Hormone group 2 (GnRH-PG-GnRH intramuscular injection with follicular fluid drainage); PS1: Cattle placenta stem cells (CPSC) group 1 (ovarian injection of 1×10^6 CPSCs with follicular fluid drainage); PS2: CPSC group 2 (ovarian injection of 6×10^6 CPSCs with follicular fluid drainage); GnRH: Gonadotrophin-releasing hormone; PG: Prostaglandin.

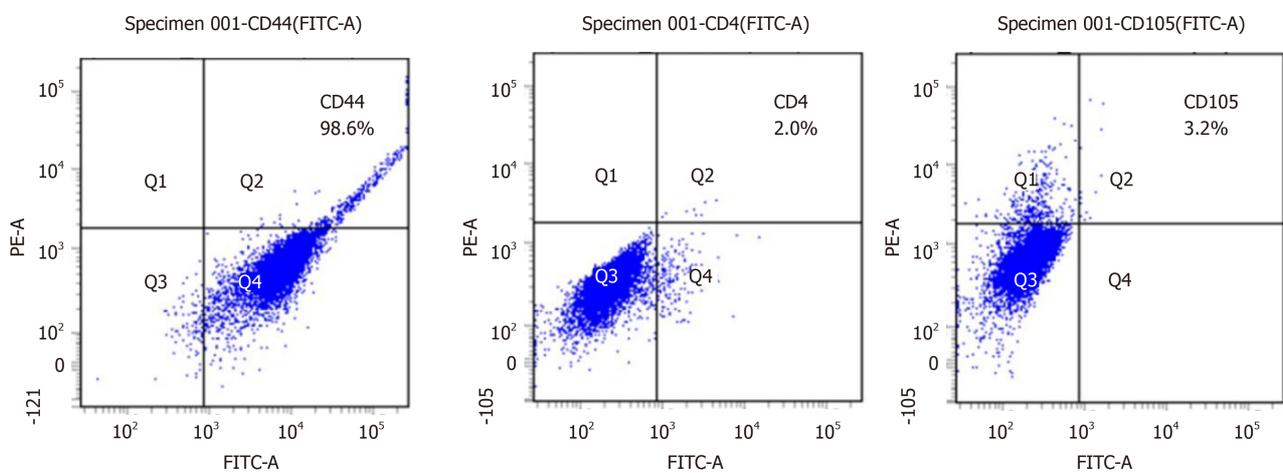


Figure 4 Flow cytometry analysis of surface marker of cattle placental stem cells. A: Cattle placental stem cells (CPSCs) expressed 98.6% CD44, which is a mesenchymal stem cell marker; B: CPSCs expressed 2.0% CD4, which is a T lymphocyte marker; C: CPSCs expressed 3.2% CD105, which is an angiogenesis-related marker.

tissues^[27,28]. Based on this characteristic, CPSCs seem to have the potential to treat OFC.

Interestingly, PSCs can modulate the maternal immune system during conception^[29] after transplantation^[19,30,31]. Besides, drainage of ovarian follicular fluid plays an essential role, because the groups with drainage had higher recovery and estrus rates in this study, consistent with previous research^[32]. Both the hormone and CPSC treatments can treat cows with OFC. The recovery rate in the H2 treatment group was consistent with previous research^[9]. However, the overall conception rates were lower in all treatment groups than shown in previous studies, although two fetuses were produced in the H2 and PS1 treatment groups^[9,32]. This outcome might be derived from the use of cows eliminated by local farmers.

From the aspect of food safety, the hormone secreted in milk might harm consumers^[33]. Hence, cell therapy seems to be an alternative method to deal with hormone problem of OFC. Growth differentiation factor-9 and bone morphogenetic protein-15 are important factors in the development of ovarian follicles^[34] and regulation of the structure of follicular or granulosa cells^[35]. Thus, it is important to acquire cystic ovarian with less expression of these factors^[36]. CPSCs can treat OFC in cows with paracrine therapeutic effect by improving tissue repair^[37] and reducing inflammation^[38]. In addition, PSCs enhance angiogenesis and cutaneous reconstruction through paracrine effects^[39-41] in animal models^[42,43]. In summary, CPSCs have the therapeutic potential of MSCs to reduce the use of hormone in cows with OFC.

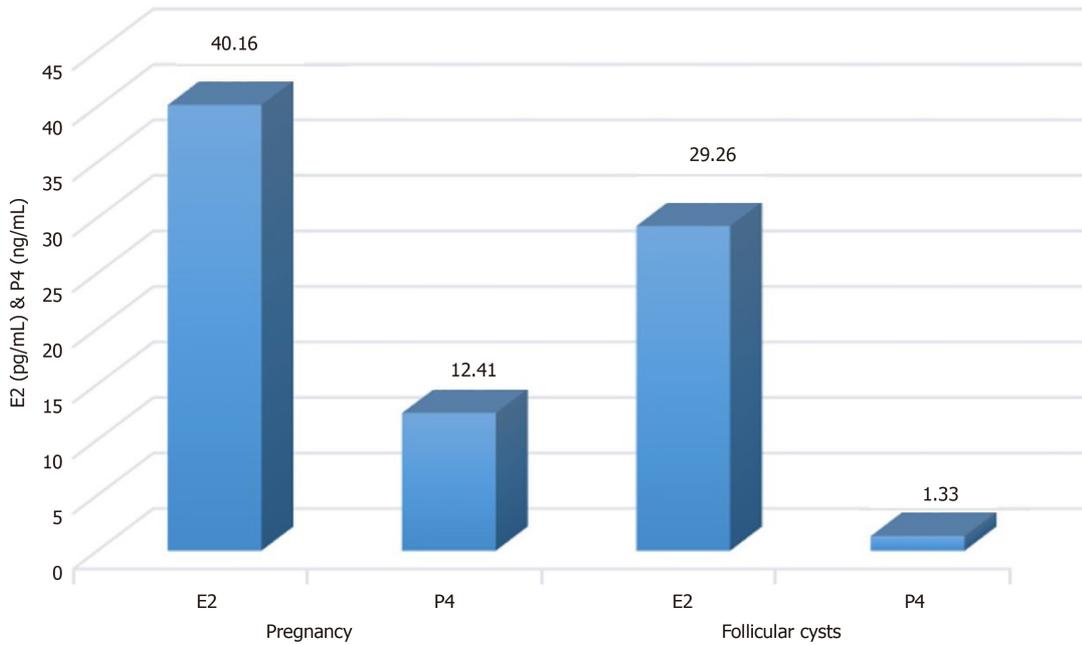


Figure 5 Concentration of sexual hormone in cows with pregnancy and ovarian follicular cysts. High concentration of estradiol (E2) and low concentration of progesterone (P4) indicated the presence of ovarian follicular cyst.

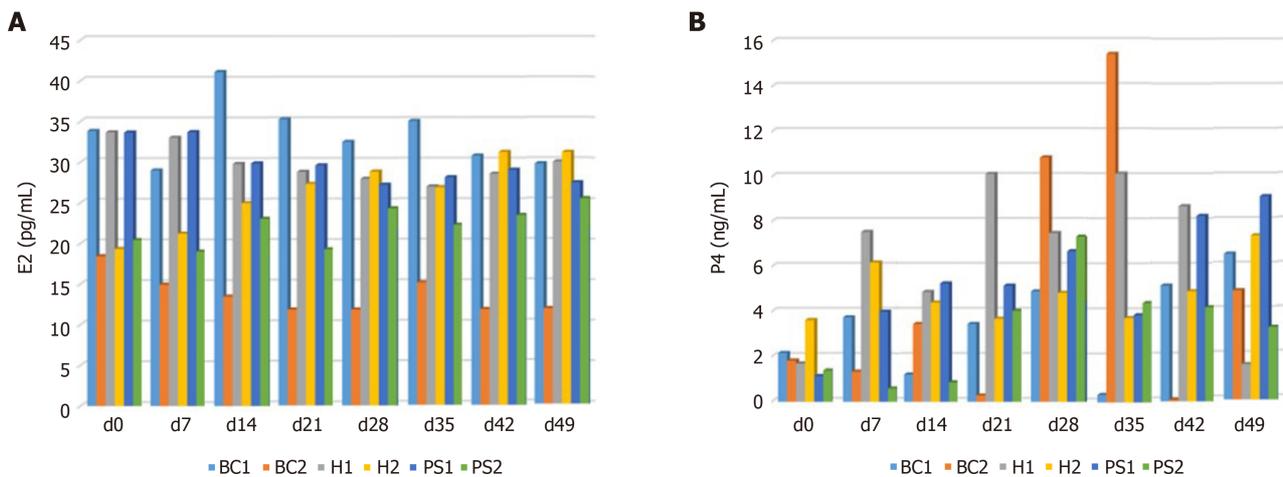
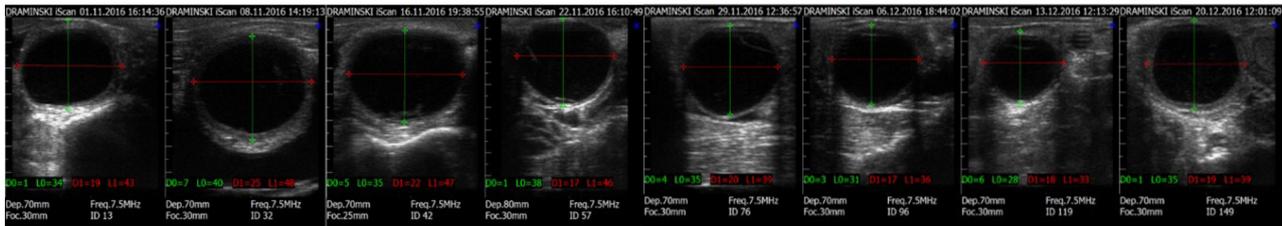


Figure 6 Comparison of estradiol and progesterone concentrations in each group. A: All hormone and cattle placental stem cell (CPSC) treatment groups had no significant difference that might originate from individual variations; B: Concentration of progesterone (P4) increased in the hormone and CPSC treatment groups, but the elevation was unstable due to individual variations. BC1: Control group; BC2: Placebo group with follicular fluid drainage and saline injection; H1: Gonadotrophin-releasing hormone (GnRH) (250 µg)-prostaglandin (PG) (500 µg)-GnRH (250 µg) hormone treatment group without drainage of follicular fluid; H2: Hormone treatment group with drainage of follicular fluid; PS1: CPSC treatment group with follicular fluid drainage and ovarian injection of 1×10^6 CPSCs; PS2: CPSC treatment group with follicular fluid drainage and ovarian injection of 1×10^6 CPSCs; E2: Estradiol.

CONCLUSION

To avoid economic loss from OFC, several treatments have been proposed to promote reproductive function and milk production. Aside from manual rupture and hormone injection, a novel therapy, CPSC transplantation, demonstrated therapeutic potential for treating OFC. CPSCs can be easily obtained from the placenta and proliferate significantly to repair tissue. Compared with hormone injection, higher recovery rate, estrus rate, and inseminated conception rate were noted in CPSC transplantation. As a consequence, this therapy can serve as an alternative approach to cure cows with OFC.

A: OFC without any treatment



B: OFC with CPSCs treatment

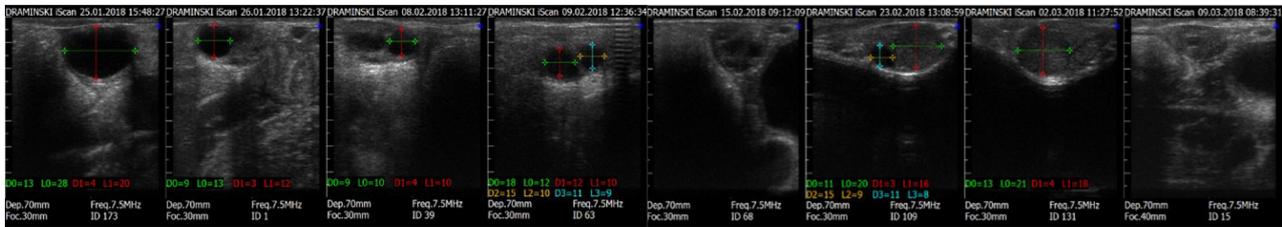


Figure 7 Ultrasonographic images of ovarian follicular cyst with and without cattle placental stem cell treatment. The series of scanning graphs were taken from weeks 1 to 7; Green and yellow lines represent transverse diameters; Red and blue lines represent longitudinal diameters. A: Ovarian follicular cyst (OFC) persisted during week 7; B: The OFC subsided progressively 7 d after injection. Cattle placental stem cell (CPSC) treatment showed the therapeutic potential.

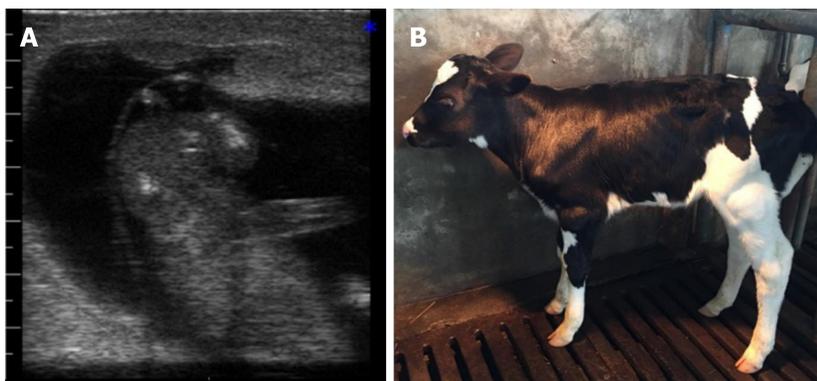


Figure 8 The heifer labored by the cow with ovarian follicular cyst after injection of one million cattle placental stem cells. A: Ultrasound scan of the fetus; B: The healthy heifer grew up with normal function.

ARTICLE HIGHLIGHTS

Research background

High humidity and temperature in Taiwan have significant effects on the reproductivity of Holstein cattle, which results in the occurrence of bovine ovarian follicular cyst (OFC). Because of economic loss from OFC, manual rupture and hormone injection have been advocated. However, these incomplete treatments decrease the conception rate and increase hormone resistance in cattle. In recent years, stem cells have been extensively utilized to repair or replace damaged tissues. Injection of stem cells might become an effective modality to treat OFC.

Research motivation

Through angiogenesis promotion and immune modulation, mesenchymal stem cells (MSCs) showed the potential in the treatment of several diseases. Besides, MSCs can be non-invasively collected from the placenta without much ethical concern. With a great source of stem cells, transplantation treatment could be conducted practically.

Research objectives

This study established the possibility of using cattle placental stem cells (CPSCs) as a treatment modality for OFC in cows.

Research methods

The cattle with OFC were divided into three groups: control (BC1 and BC2), hormone (H1 and H2), and CPSC (PS1 and PS2) treatment groups. In the hormone treatment group, the cows were given gonadotrophin-releasing hormone (GnRH)-prostaglandin-GnRH injection with or without drainage of follicular fluid. In the CPSC treatment groups, CPSCs were isolated from the placenta. The cows were given ovarian injection of 1×10^6 or 6×10^6 CPSCs with drainage. Then OFC was scanned by ultrasound once a week for a total of seven times. The concentrations of estradiol and progesterone in serum were tested in the same period. The estrus cycle was analyzed by food intake and activity. If estrus was detected, artificial insemination was conducted. The cow was monitored by ultrasound for confirmation of pregnancy.

Research results

After 7 d of culture, CPSCs were successfully isolated from placental pieces. CPSCs proliferated significantly every 24 h and highly expressed MSC markers, such as CD44. In an ultrasound study, more subsided OFCs were observed with drainage of follicular fluid. The recovery rates were 0%, 50%, 50%, 75%, 75%, and 75% in BC1, BC2, H1, H2, PS1, and PS2, respectively. The estrus rate was 0%, 100%, 25%, 75%, 75%, and 75% in BC1, BC2, H1, H2, PS1, and PS2, respectively. Two fetuses were born in H2 and PS1.

Research conclusions

Cows with CPSC injection achieved higher recovery, estrus, and inseminated conception rates. This approach shows efficacy in treating cows with OFC.

Research perspectives

CPSC injection could serve as an alternative treatment for OFC. In the future, other reproductive disorders might also be investigated with stem cell therapy.

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