Basic Study

Application of cattle placental stem cells for treating ovarian follicular cyst

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Institutional animal care and use

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

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 imbalance in hormones from the hypothalamus and pituitary gland. This abnormal imbalance in hormones from the hypothalamus and pituitary gland. This abnormal imbalance in hormones from the hypothalamus and pituitary gland. This abnormal imbalance in hormones from the hypothalamus and pituitary gland. This abnormal
condition causes ovulation failure and decreases the profits for farmers\cite{11}. 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\cite{11}. Consequently, an alternative approach is using hormones, such as human chorionic gonadotrophin\cite{16} and gonadotrophin-releasing hormone (GnRH)\cite{20}. Unfortunately, this treatment increases hormone resistance or has no effect on cattle\cite{20}.

Recently, the use of stem cells to repair or replace damaged tissue has been extensively studied\cite{11}. Stem cells secrete growth factors and anti-inflammatory factors\cite{11} and reduce fibrosis\cite{20}. Several studies have reported the success of stem cells for treating retinal\cite{8}, liver\cite{14}, and cardiac diseases\cite{15}. On the other hand, the placenta can be non-invasively collected as great sources of stem cells without much ethical concern\cite{11}. Many placental stem cells (PSCs) can be identified by the expression of mesenchymal stem cells (MSCs) and pluripotency markers\cite{21}. These cells can be differentiated into mesodermal-relative cells\cite{11}. In addition, PSCs have therapeutic properties for the management of several diseases such as wound healing\cite{20}, diabetes\cite{16}, lung fibrosis\cite{20}, among others\cite{21,22}. 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 (αMEM 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% CO\textsubscript{2}, 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 × 10⁶ 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 × 10⁵ 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 × 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 μg)-prostaglandin (PG) (500 μg)-GnRH (250 μg) 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 × 10⁵ CPSCs with follicular fluid drainage. The PS2 CPSC treatment group was given ovarian injection of 6 × 10⁵ 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.
Peng SY et al. Treatment of OFC with stem cells

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.
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. 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. Our research showed that CPSCs had strong properties of proliferation and expression of MSC markers, similar to previous studies. 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 $1 \times 10^6$ CPSCs with follicular fluid drainage); PS2: CPSC group 2 (ovarian injection of $6 \times 10^6$ CPSCs with follicular fluid drainage); GnRH: Gonadotrophin-releasing hormone; PG: Prostaglandin.

![Flow cytometry analysis of surface marker of cattle placental stem cells](image_url)

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.

Based on this characteristic, CPSCs seem to have the potential to treat OFC. Interestingly, PSCs can modulate the maternal immune system during conception after transplantation. 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. Both the hormone and CPSC treatments can treat cows with OFC. The recovery rate in the H2 treatment group was consistent with previous research. 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. 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. 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 and regulation of the structure of follicular or granulosa cells. Thus, it is important to acquire cystic ovarian with less expression of these factors. CPSCs can treat OFC in cows with paracrine therapeutic effect by improving tissue repair and reducing inflammation. In addition, PSCs enhance angiogenesis and cutaneous reconstruction through paracrine effects in animal models. 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 $1 \times 10^6$ CPSCs; PS2: CPSC treatment group with follicular fluid drainage and ovarian injection of $1 \times 10^6$ 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.
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.
Peng SY et al. Treatment of OFC with stem cells

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 $1 \times 10^6$ or $6 \times 10^6$ 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 BCI, 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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