

Method for isolating pure bovine gonadotrophs from anterior pituitary using magnetic nanoparticles and anti-gonadotropin-releasing hormone receptor antibody

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ABSTRACT. No methods are currently available for rapidly isolating gonadotrophs from the anterior pituitary (AP) in any species. We developed a method for preparing pure bovine gonadotrophs from a heterogeneous AP cell mixture by magnetic separation and our original antibody against the N terminus of bovine gonadotropin-releasing hormone receptor (GnRHR). A bovine AP cell mixture was incubated with the anti-GnRHR antibody, anti-dextran antibody-conjugated secondary antibody and dextran-coated magnetic nanoparticles for magnetic isolation. Approximately 5.2×10^6 cells were isolated per AP of Japanese Black heifers (26 months of age) and cultured, and confocal microscopy confirmed to be GnRHR- and luteinizing hormone-positive, corresponding to a purity of 100%. Approximately 44.5 μ g of total protein was extracted from the pure gonadotrophs per AP.

KEY WORDS: EasySep, gonadotroph, luteinizing hormone, ruminant

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Gonadotrophs in the anterior pituitary (AP) are important cells; however, the AP has a heterogeneous cell population, and gonadotrophs constitute only 10 to 15% of all cells in the AP and are scattered among other cell types in rats and humans [2]. To clarify the mechanisms of action of hormone-secreting AP cells, previous studies have tried to purify specific cell types from heterogeneous AP cell mixtures; however, these attempts have resulted in failure to obtain gonadotrophs in rats [12] or in a low purity of gonadotrophs in sheep [4].

We recently developed a highly specific antibody that recognizes the extracellular region at the N terminus of bovine gonadotropin-releasing hormone receptor (GnRHR) [7]. We could obtain pure bovine gonadotrophs utilizing the antibody for fluorescence-activated cell sorting analysis of bovine AP cell mixture [7]. However, the slow speed of this method was unsatisfactory, since the system could only process one sample at a time.

The EasySep magnetic separation system (Stemcell Technologies, Vancouver, Canada) is a simple method for the simultaneous isolation and purification of cells using a strong magnet. Recently, microglia were purified from murine brain using the EasySep magnetic separation method [6]. In the present study, we developed a new method to obtain pure bovine gonadotrophs from AP using EasySep and the anti-GnRHR antibody.

Experiments were approved by the Committee on Animal Experiments of Yamaguchi University. Fluorescein iso-

thiocyanate (FITC) was conjugated to the anti-GnRHR antibody using the Surelink FITC Labeling kit (Kirkegaard & Perry Laboratories, Baltimore, MD, U.S.A.) according to the manufacturer's protocol. AP tissue was obtained from post-pubertal Japanese Black heifers (n=6, 26 months of age, various stages of the estrous cycle) from a local slaughterhouse in Yamaguchi prefecture in Japan and transported to the laboratory as described previously [10]. The experiment was repeated six times with each sample. AP cells were prepared as previously described [11]. Briefly, AP tissue was cut into small pieces (less than 1 mm³) that were centrifuged (450 \times g for 5 min at room temperature) and resuspended in 10 ml of HEPES buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄ · 12H₂O, 10 mM glucose, 25 mM HEPES and 36 μ M CaCl₂ · 2H₂O). The washing step was repeated five times. The tissue was then incubated at 37°C in 10 ml of HEPES buffer containing 11,200 U collagenase (032-22364; Wako, Osaka, Japan) and 1% bovine serum albumin (Wako) for 45 min while pipetting every 5 min. After washing with 2% fetal bovine serum (FBS) in HEPES buffer (2% FBS), the cells were resuspended in 1 ml of the same solution. Cell suspensions were passed through a 200- μ m filter (Filcons cup; As One Corporation, Osaka, Japan), followed by a 30- μ m filter (CellTrics, Partec GmbH, Gortitz, Germany) to obtain a single-cell preparation. The yield was $1.9 \times 10^7 \pm 0.9 \times 10^7$ cells per AP, as determined using a cell counter (Model TC20; Bio-Rad, Hercules, CA, U.S.A.). Cell viability was >95%, as confirmed by Trypan Blue exclusion.

We used the EasySep Other Cells FITC Positive Selection kit (18558, Stemcell Technologies) according to the manufacturer's instructions. Dissociated AP cells were resuspended in 1 ml of 2% FBS. We compared non-fixed cells and cells fixed with a non-toxic formulation for preserving proteins (CellCover; AI Anacyte Laboratories UG, Hamburg, Germany). Half of the cell suspension (0.5 ml) was transferred to a 12 \times 75 mm polystyrene tube, and cells were fixed for 2 min

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at room temperature with CellCover, washed three times with 2% FBS and incubated for 15 min at room temperature in the dark with 1.5 μg FITC-labeled anti-GnRHR antibody dissolved in 0.5 ml of 2% FBS. The remaining 0.5 ml of cell suspension was transferred to a separate tube and incubated for 15 min at room temperature with 1.5 μg of FITC-labeled anti-GnRHR antibody without fixation. After the primary antibody reaction, fixed or non-fixed cells were incubated for 15 min at room temperature with 50 μl of the anti-dextran antibody-conjugated anti-FITC antibody. The reaction mixture was incubated for 10 min with 25 μl of dextran-coated magnetic nanoparticles; the reaction mixture volume was made up to 2.5 ml with 2% FBS, with gentle mixing by pipetting. The tube was placed on the EasySep magnet for 5 min at room temperature, and the cell suspension was decanted in a continuous motion to pour off the supernatant fraction (discarded solution) into another separate polystyrene tube, allowing the magnetically labeled (i.e., isolated) cells to be retained within the magnetic field. The discarded solution was transferred to a low-protein-binding microtube (Proteosave SS; Sumitomo Bakelite, Tokyo, Japan), which was centrifuged at 450 $\times g$ for 5 min at room temperature to obtain the non-isolated cell pellet. The pellet was resuspended in 5,000 μl of 2% FBS, and 40 μl of the cell suspension was loaded into a μ -Slide VI microscopy chamber (Ibidi, Planegg, Germany; each lane was 17 mm long and 3.8 mm wide, with a thickness of 0.4 mm) for confocal microscopy observation. The polystyrene tube containing the isolated cells was removed from the magnet, and 2.5 ml of 2% FBS was added to the tube. After mixing by pipetting, the tube was replaced on the magnet for 5 min and inverted to pour off the supernatant fraction. This washing step was repeated, and isolated cells were resuspended in 500 μl of 2% FBS and transferred to another low-protein-binding microtube; 40 μl was then loaded into another lane of the same μ -Slide VI microscopy chamber for confocal microscopy observation, whereas 20 μl was used for both cell counts and Trypan Blue exclusion. The remaining cell suspension was centrifuged at 450 $\times g$ for 5 min at room temperature, and the pellet was stored at -80°C until western blot analysis.

Isolated and non-isolated fixed cells in the μ -Slide VI microscopy chamber were visualized by confocal microscopy (LSM710; Carl Zeiss, Göttingen, Germany). Fluorescence and differential interference contrast images were acquired using a charge-coupled device (CCD) camera to prepare single-plane confocal images (ZEN2010; Carl Zeiss).

Isolated cell pellets were thawed and homogenized by pipetting and vortexing in 18 μl Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, U.S.A.) containing protease inhibitors (Halt protease inhibitor cocktail; Thermo Fisher Scientific). The total amount of protein in 3 μl of each sample was measured with a bicinchoninic acid kit (Thermo Fisher Scientific), and 2.5 μg of total protein was analyzed alongside bovine luteinizing hormone (LH) [AFP11118B; National Hormone and Pituitary Program (NHPP), Bethesda, CA, U.S.A.] and protein extracted from the AP tissue of a heifer for size comparison. Western blotting was carried out using a previously described protocol [7]

using a mouse monoclonal anti-LH antibody (1:40,000 dilution) [9] that recognizes the bovine LH β subunit and does not cross-react with other bovine pituitary hormones [5]. Protein bands were visualized using a chemiluminescence kit (ECL Prime, GE Healthcare, Amersham, U.K.) and CCD system (LAS-3000 Mini; Fujifilm, Tokyo, Japan). Antibodies were removed from the polyvinylidene difluoride membrane, which was blocked and incubated with mouse monoclonal anti- β -actin antibody (1:50,000 dilution; Sigma-Aldrich, St. Louis, MO, U.S.A.). Immunoreactivity was visualized using a chemiluminescence kit and CCD system.

Isolated cells were cultured with the above-mentioned FITC Positive Selection method. However, confocal microscopy detected the FITC signal on the surface of approximately 5% of cells cultured for 3 days, making them inappropriate for further confocal microscopy analysis. We therefore used the EasySep Other Cells Biotin Positive Selection kit (18559, Stemcell Technologies) for confocal microscopic observation of isolated cells. Anti-GnRHR antibody was labeled with the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce Biotechnology, Rockford, IL, U.S.A.) according to the manufacturer's protocol. Dissociated AP cells from AP tissue of post-pubertal Japanese Black heifers ($n=4$, 26 months of age) were prepared by the protocol described above. Dissociated AP cells were resuspended in 1 ml of 2% FBS dissolved in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, U.S.A.). The cell suspension was incubated for 15 min at room temperature with 3 μg of biotin-labeled anti-GnRHR antibody. After the primary antibody reaction, cells were incubated for 15 min at room temperature with 100 μl of anti-dextran antibody-conjugated streptavidin. The reaction mixture was incubated for 10 min with 50 μl of dextran-coated magnetic nanoparticles, and cells attached to the anti-GnRHR antibody were isolated with a magnet using the protocol described above and resuspended in 1 ml DMEM containing 1% nonessential amino acids (100 \times ; Gibco), 100 IU/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 10% horse serum (Gibco) and 2.5% FBS (Gibco). The cell suspension (150 μl per lane) was loaded into six lanes of a μ -Slide VI microscopy chamber and cultured at 37°C in a humidified atmosphere of 5% CO_2 for 84 hr, while 20 μl of the cell suspension was used for cell counts with the same cell counter and Trypan Blue exclusion. The cultured cells in the μ -Slide VI microscopy chamber were treated with 4% paraformaldehyde in PBS for 3 min and then with 0.1% Triton X-100 in PBS for 3 min. Immunocytochemistry was carried out as previously described [7] using the same primary antibodies for GnRHR and LH as well as Alexa Fluor 546 goat anti-mouse IgG (Thermo Fisher Scientific), Alexa Fluor 647 goat anti-guinea pig IgG (Thermo Fisher Scientific) and 4',6'-diamino-2-phenylindole (DAPI; Wako). Cells were visualized by confocal microscopy.

Differences in total cell or total protein yield per AP between fixed isolated cells and non-fixed isolated cells were compared with the non-paired *t*-test. The level of significance was set at $P<0.05$. Data are expressed as the mean \pm standard error of the mean.

The total cell yields were $5.2 \times 10^6 \pm 2.0 \times 10^6$ and 7.6

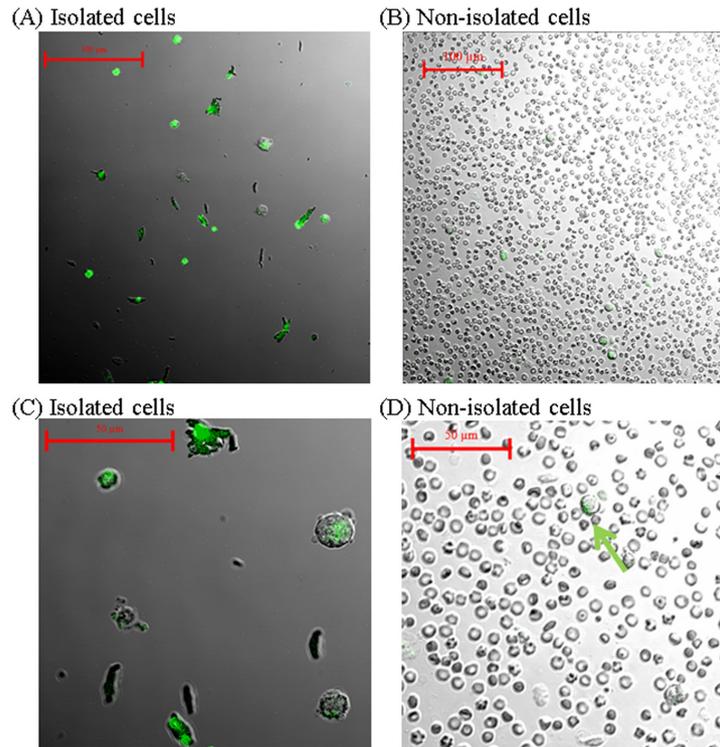


Fig. 1. Representative merged FITC and differential interference contrast images of isolated (A, C) and non-isolated (B, D) cells at low (A, B; scale bars=100 μ m) and high (C, D; scale bars=50 μ m) magnification, obtained by confocal microscopy. FITC-labeled anti-GnRHR antibody was detected on the surface of all isolated cells that were not treated with Triton X-100. Note that all cells were floating and moving inside the microscopy chamber (17 mm long, 3.8 mm wide and 0.4 mm thickness). The green arrow in panel D indicates FITC-positive cells that were not isolated.

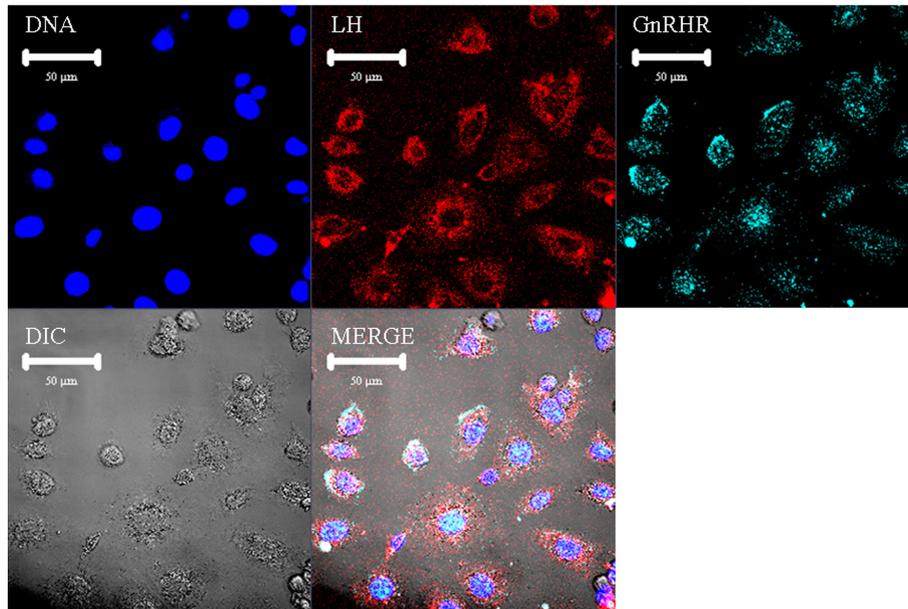


Fig. 2. Immunocytochemical analysis of gonadotrophs cultured for 84 hr after isolation from heterogeneous bovine AP cells in the microscopy chamber, as visualized by laser confocal microscopy. Cells were labeled with antibodies against LH (red) and GnRHR (light blue); Nuclear DNA was counterstained with DAPI (dark blue), and cell morphology was visualized by differential interference contrast (DIC) imaging. The MERGE is an overlay of the four panels. Cells were treated with Triton X-100, and anti-GnRHR antibody was detected in both the surface and cytoplasm of all isolated cells. Note that all cells were attached to the microscopy chamber. Scale bars=50 μ m.

$\times 10^6 \pm 1.1 \times 10^6$ cells per AP using the protocols for preparing fixed and non-fixed gonadotrophs, respectively; the total protein yields were 44.5 ± 7.0 and $39.1 \pm 9.2 \mu\text{g}$ per AP, respectively. There were no significant differences in the values obtained for fixed and non-fixed cells. The viability of the non-fixed cells was $>90\%$.

We observed more than 10^3 cells for each AP by confocal microscopy. All isolated cells were positive for the FITC signal resulting from the binding of the FITC-labeled anti-GnRHR antibody to the surface of gonadotrophs (Fig. 1A). In contrast, most non-isolated cells were negative for the FITC signal (Fig. 1B), although a few immunoreactive cells were detected.

The western blot analysis of LH expression revealed an LH-immunoreactive protein band with an apparent molecular weight of 19 kDa in the protein extract from AP tissue, NHPP LH and isolated fixed cells; however, only a weak signal was observed in the protein extract from isolated non-fixed cells.

The total cell yields were $7.5 \times 10^6 \pm 1.2 \times 10^6$ cells per AP after isolation by biotin-positive selection. Cell viability was $>90\%$. We observed more than 10^3 cells for each AP by confocal microscopy (Fig. 2) and confirmed that all of the cells expressed GnRHR and LH. Thus, the purity of gonadotrophs was estimated as 100%.

Gonadotrophs are larger than other secretory cells in the rat AP [3]. The FITC-positive cells observed in this study were larger than those that were negative for FITC, and their shape and size were very similar to those of bovine gonadotrophs that were immunoreactive to anti-LH and anti-GnRHR antibodies in previous studies [1, 7]. The western blot analysis indicated that the isolated cells contained LH. We therefore conclude that the FITC-positive cells are bovine gonadotrophs, which were purified in the present study by a novel method.

We obtained 10^7 heterogeneous cells per AP and isolated 10^6 gonadotrophs per AP from the heterogeneous cells. Gonadotrophs constitute only 10 to 15% of all AP cells [2]; thus, our yield was consistent with estimated values.

Dairy cows can secrete LH from AP only weakly in the postpartum period [8], however, the precise mechanisms remain to be clarified. This method may be useful for preparing pure gonadotroph samples from dairy cows.

In conclusion, the method described in this study provides a novel and simple method for the rapid simultaneous purification of bovine gonadotrophs from heterogeneous AP cell mixture using the EasySep magnetic separation method and our original anti-GnRHR antibody.

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