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Tissue localization of GM-CSF receptor in bovine ovarian follicles and its role on glucose uptake by mural granulosa cells

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ABSTRACT

The granulocyte-macrophage colony stimulating factor (GM-CSF) is a multifunctional cytokine implicated in proliferation, differentiation, and activation of several cell types including those involved in hematopoiesis and reproduction. In the present study, the expression of the α- and β-subunit genes of GM-CSF receptor during follicular development in cattle was assessed. The spatial association of α- and β-subunits of GM-CSF with follicle stimulating hormone receptor (FSHR) and 3β-hydroxysteroid dehydrogenase (3β-HSD), and the temporal associations with gene expression of hexose transporters (GLUTs) in granulosa cells of cattle were also evaluated. The effect of GM-CSF on the functionality of hexose transporters was also determined in an *in vitro* primary culture of granulosa cells. The spatial association of subunits of the GM-CSF receptor with 3β-HSD and FSHR suggests a potential steroidogenic regulation of GM-CSF in granulosa cells. Immunodetection of GLUTs and uptake kinetic assays confirmed expression and functionality of these genes for hexose transporters in granulosa cells of cattle. Treatment of granulosa cells with GM-CSF, FSH or insulin- like growth factor-I (IGF-I) alone increased 2-deoxyglucose (DOG) or 3-0-methylglucose (OMG) uptake; however, when cells were treated with various combination of these factors there were no additive effect. Unexpectedly, the combination of GM-CSF and FSH decreased DOG uptake compared to FSH treatment alone. Thus, the expression pattern of GM-CSF receptor subunit genes during follicle development in cattle and promotion of DOG and OMG uptake in granulosa cells indicate a role for GM-CSF, FSH and/or IGF-I alone in regulating granulosa cell metabolic activity, specifically by promoting glucose uptake.

1. Introduction

The granulocyte-macrophage colony stimulating factor (GM-CSF) is a multifunctional cytokine initially identified as a regulator of proliferation, differentiation, and activation of myeloid hematopoietic cells (Ruef and Coleman,

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1990). The GM-CSF receptors are heterodimers comprising a cytokine-specific α-subunit and a common β-subunit (βc), which is the principal signal transducing subunit shared by interleukin IL-3 and IL-5 receptors. The GM-CSF receptors have been highly characterized in the hematopoietic cell line, as well as in other cell types including neural, neoplastic, and embryonic (Metcalf et al., 1990; Baldwin et al., 1989, 1993; Vilanova et al., 2003; Zambrano et al., 2010; Peralta et al., 2013). In reproductive tissues, selective expression of the GM-CSF receptor genes in the uterus and ovary have also been reported and temporal activation and spatial association have suggested a potential regulatory function during reproductive cycles (Brännström et al., 1994; Robertson et al., 1996).

Previous studies have reported that expression of GM-CSF genes in theca, granulosa and luteal cells is associated to follicular development, ovulation and luteinization in humans and rodents (Gilchrist et al., 2000; Zhao et al., 1995; Tamura et al., 1998). In humans, the GM-CSF receptor α-subunit is located in various cell types throughout the ovary, where gene expression increases in theca externa and granulosa cells as follicles develop (Zhao et al., 1995). Also in humans, there is prominent expression of GM-CSF receptor β-subunit genes within the vascular regions of the ovary, including the corpus luteum (CL) and theca layer (Zhao et al., 1995; Jasper et al., 1996).

Changes in estrogen and progesterone concentrations regulate the production of GM-CSF which suggests a potential regulatory function during the estrous cycle of mice (Robertson et al., 1996). Thus, selective expression of the GM-CSF gene in the human and mouse ovary and uterus and its steroidogenic regulation during the estrous cycle suggest an autocrine/paracrine role in folliculogenesis. A comparable role may also be exerted in ovarian physiology of cattle; however, the tissue-specific gene expression pattern and function of the GM-CSF complex in the ovary in cattle have not been elucidated.

In mammals, there are two super-families of hexose transporters: the co-transporters glucose and sodium, and the facilitative hexose transporters or glucose transporters (GLUTs). The GLUTs are capable of transporting hexoses across the plasma membrane by facilitated diffusion (Uldry and Thorens, 2004). These transporters are a family of membrane proteins 45–55 kDa in size of which several have been identified as functional isoforms (GLUT 1–14; Manolescu et al., 2007; Chen et al., 2015). Upon binding to the receptor, GM-CSF promotes glucose uptake through PI 3-kinase/Akt pathway via translocation of GLUT 1 to the plasma membrane (Zambrano et al., 2010).

It has been described that GM-CSF increases the uptake of sugars and vitamin C, through facilitative hexose transporters in neutrophils, human monocyte L-60 and HEK 293 cell lines (Vera et al., 1995; Angulo et al., 1998; Zambrano et al., 2010), sperm and embryos of mice (Zambrano et al., 2001; Rauch et al., 2004; Rodríguez-Gil et al., 2007; Robertson et al., 2001). It has been suggested that FSH increases glucose uptake during oocyte meiotic maturation (Downs et al., 1996; Downs and Utecht, 1999; Roberts et al., 2004; Sutton et al., 2003). In addition, FSH increases facilitated diffusion in murine granulosa cells during all stages of follicular development (Boland et al., 1994). IGF-I increase glucose oxidation in mature granulosa cells of pigs (Weber and La Barbera, 1988). Moreover, there is evidence that FSH and IGF-I positively modulate the effect of GLUT 1 on glucose uptake in granulosa cells of rats and mice (Kodaman and Behrman, 1999; Zhou et al., 2000) and that IGF-I may have an important role on GLUT 3 in mouse muscle cells (Bilan et al., 1992). GLUT4 is known as the main facilitator responsible for incorporation of insulin-stimulated glucose (Bell et al., 1990). Whether or not GM-CSF alone or in combination with FSH or IGF-1 can potentiate the effect of this glucose uptake transporter in bovine granulosa cells remains unknown.

It has been recently reported (Peralta et al., 2013) that GM-CSF receptor subunit genes are expressed in cumulus cells of cattle and that exogenous GM-CSF enhances cumulus cell expansion in *in vitro* matured cumulus-oocyte complexes (COC). Hence, evaluation of the molecular and functional characterization of GM-CSF receptors will contribute to a greater understanding of the metabolic activity of ovarian follicles during complex process of folliculogenesis in cattle. Main goals of the present study in cattle were: (1) determine the expression of the α- and β-subunit genes of the GM-CSF receptor from primordial to antral stage follicles, (2) determine the spatial association of α- and β-subunits of GM-CSF with follicle stimulating hormone receptor (FSHR) and 3β-hydroxysteroid dehydrogenase (3β-HSD), and its temporal association with expression of the GLUTs gene in isolated granulosa cells of antral follicles, and (3) determine the effect of GM-CSF on the functionality of hexose transporters in an *in vitro* primary culture of granulosa cells collected from antral follicles.

2. Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless stated otherwise.

2.1. Immunohistochemistry

Cattle ovaries were obtained from a local abattoir and transported to the laboratory immersed in 0.85% saline supplemented with 100 mg/mL of streptomycin and 80 mg/mL sodium penicillin G at a temperature of 35–38 °C within 30 min of collection. Ovaries were fixed in 4% paraformaldehyde buffered in PBS, embedded in paraffin and serially sectioned at 6-μm intervals for immunohistochemical analyses. Tissue sections were mounted on adhesive coated slides and incubated overnight at 37 °C. Mounted tissues were deparaffinized in xylene and rehydrated in serial alcohol solutions. Slides were subjected to an unmasking protocol by microwave antigen retrieval at 700 W for 1 min in sodium citrate solution (10 mM, pH 6.0). Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxidase diluted in 0.1 M PBS for 5 min at room temperature. Slides were then rinsed two times in PBS and immersed in blocking solution (2.5% horse serum, 5% BSA, 0.3% triton X-100, pH 7.4) for 60 min at room temperature. Tissues were probed by incubating overnight at room temperature with anti-α-GM-CSF receptor antibody (1:200; C-18, Cat. # sc-690 Santa Cruz Biotechnology, CA, USA) raised against the carboxyl termi-

nal of the α-GM-CSF receptor subunit. Serial slides were probed similarly by incubating with anti-β-GM-CSF receptor antibody (1:200; N-20, Cat. # sc-1318; Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against the amino terminals of the β-GM-CSF receptor subunit. These GM-CSF antibodies have been validated in previous studies (Rodríguez-Gil et al., 2007; Peralta et al., 2013). After two washes in PBS, the binding of the primary antibodies were detected using pan-specific secondary antibody conjugated to horseradish-peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by incubating for 10 min at room temperature. Immune complexes were visualized using 3,3′-diaminobenzidine (DAB) substrate for 5 min or until the signal became visible. Probed sections were then counterstained with hematoxylin and dehydrated in serial alcohol solutions. Sections were mounted with permount (Fisher Scientific, NH, USA) under coverslips. The following procedural controls were performed on neighboring sections: (1) replacement of the primary antibody with non-immune serum, (2) replacement of the secondary antibody with non-immune serum, and (3) omission of both primary and secondary antibodies, followed by incubation in DAB alone. Digital photos of tissue sections were obtained using bright microscopy (Olympus Vanox-T, Tokyo, Japan) and staining in different slides was compared simultaneously. Primordial, primary, secondary and antral follicles were categorized using slight modification from histological criteria previously described (Braw-Tal and Yossefi, 1997). A primordial follicle was defined as an oocyte surrounded by a single layer of flattened granulosa cells (less than 10 cells). Primary follicles were classified as follicles with one or more cuboidal granulosa cells (10–40) arranged in a single layer around an oocyte. Secondary follicles were classified as oocytes surrounded by two or more layers of cuboidal granulosa cells at any point around a follicle that lacked an antrum. Antral follicles were classified as oocytes surrounded by more than six layers of granulosa cells and antrum formation.

2.2. Collection of mural granulosa cells from antral follicles

Follicles were dissected from ovarian tissue under stereomicroscope and classified in atretic and non atretic according to Kruip and Dieleman (1982). Only the non atretic follicles with uniform bright appearance, an extensive and very fine vascularization and non- free floating particles in the follicular fluid were considered to cut in half and scrap to get the granulosa cells. Follicles were collected from a total of 50 ovaries and were pooled according to size (<4 mm, 4–8 mm, >8 mm, $n = 50$/group) in plastic 60 mm Petri dishes in Dulbecco's modified Eagle with Ham F-12 (DMEM-F12) supplemented with sodium 1.2 g/L bicarbonate, 1 mM piruvate, 2 mM glutamine and 1% ITS, 100 IU/mL penicillin, 50 mg/mL streptomycin, 0.25 μg/mL fungizone (Quirk et al., 2004) and sectioned to obtain granulosa cells and oocytes. The granulosa cell layer was removed by gentle rubbing with a glass Pasteur pipette, previously modified by heat sealing the tip into a rounded smooth surface. Cells were centrifuged at 300*g* in PBS and 0.1% BSA and fixed in histochoice-ethanol (4:1) solution or resuspended in lysis buffer and stored at 4 °C for immunofluorescence and western blot analyses, respectively.

2.3. Immunofluorescence

Fixed granulosa cells were placed in poly-L-lysine-treated glass slides and dried at room temperature. Cells were permeabilized and blocked in a solution containing 0.1 M PBS, 1% BSA, 5% skim milk and 0.3% triton-100 for 60 min at room temperature. Thereafter, slides were immersed in the same blocking solution (without triton X-100) containing the primary polyclonal antibodies in a humid chamber overnight at 4 °C. The dilutions used for the primary rabbit polyclonal antibodies were: anti-α GM-CSF (1:200, C-18, Cat. # sc-690), anti-β GM-CSF (1:200, N-20, Cat. # sc-676), anti-FSHR (1:100, N-20, Cat.# sc-7798), anti-3β-HSD (1:500, P-18, Cat.# sc-30820), (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Additional slides were incubated with anti-GLUT1 (1:100, Cat.# GT11-S), anti-GLUT2 (1:100, Cat.# GT21-S), anti-GLUT4, (1:100, Cat.# GT41-S; Alpha Diagnostics, TX, USA) and anti-GLUT3 (M-20): Cat.# sc-7582, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes with PBS, cells were incubated with anti-rabbit, anti-goat or anti-mouse IgG (1:300 in blocking buffer) conjugated with Alexa Fluor 488 and 594 nm (Molecular Probes, CA, USA). Cells were counterstained with propidium iodine (PI) for GLUTs immunodetection. Then cells were washed in PBS and mounted under coverslips in fluorescence mounting media (DAKO Laboratories, Denmark). The following procedural controls were performed on neighboring sections: (1) replacement of the primary antibody with non-immune serum, (2) replacement of the secondary antibody with non-immune serum, and (3) omission of both primary and secondary antibodies, followed by incubation in PI alone. Samples were examined under confocal microscope and photos were obtained using photomicroscopy (Olympus Fluoview 1000, Japan). Staining in different slides was compared simultaneously. The overall presence and distribution of positively labeled tissue were examined by light microscopy at low magnification (400×); high magnification was used for analysis of cellular localization (1000×). A minimum of 15 fields were visualized for each slide for the assessment of positive cells.

2.4. Western blot

Granulosa cells were centrifuged at 300*g* with PBS and homogenized in cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% triton-X-100, 1% deoxycholate, 0.1% SDS). Homogenates were centrifuged at 13,500 x *g* for 5 min; thereafter, supernatants were transferred into a new tube. Total protein concentrations were determined using a bicinchoninic acid (BCA) kit (Pierce, IL, USA) according to the manufacturing's instructions. Proteins were denatured by mixing the samples 1:1 (v/v) with Laemmli buffer (BioRad Laboratories, CA, USA) and heating to 98 °C for 5 min. Aliquots containing 20 μg of total protein were loaded in each lane and separated by SDS-PAGE in 10% gels (BioRad Laboratories, CA, USA) at 125 V. Proteins were then transferred onto PDVF membranes by electroblot-

ting at 100 V for 1 h. After blocking for 1 h in blocking buffer (LI-COR Corporation, NE, USA), membranes were probed for anti-α GM-CSF (N-20), anti-β GM-CSF (C-18), FSHR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:400 for 1 h at room temperature. Additional membranes blotted with protein from the same samples were processed identically with anti-GLUT1, anti-GLUT2, anti-GLUT4, (1:100, Alpha Diagnostics, TX, USA) and anti-GLUT3 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for 1 h at room temperature. Primary antibodies were diluted in 0.1% tween-20 in blocking buffer (LI-COR Corporation, Lincoln, Nebraska, USA). After four, 5 min washes in 0.1% Tween-20 in PBS, membranes were incubated in the appropriate anti-mouse or anti-rabbit IRDye-conjugated secondary antibody (LI-COR Corporation, Lincoln, Nebraska, USA) diluted 1:5000 in 0.1% Tween-20 in blocking buffer for 30 min.

2.5. Primary culture of granulosa cells from antral follicles

The collection of granulosa cells was performed similar to the method previously described (Section 2.2). In brief, only antral follicles 4–8 mm ($n = 60$) in diameter were dissected from ovarian tissue were collected from 70 ovaries under the microscope in 60-mm plastic petri dishes containing DMEM-F12 modified medium and sectioned to obtain granulosa cells and oocytes. The cells were collected using a glass Pasteur pipette as previously described (Section 2.2). The DMEM-F12 medium containing the granulosa cells were centrifuged twice at 300*g* for 5 min at room temperature. Number of cells were estimated by hemocytometer and cells were seeded in 12-multiwells plastic culture plates at a concentration of 1×10^6 per well in 1 mL of DMEM-F12 medium and cultured for 20 h at 37 °C under a humidified atmosphere containing 5% CO_2.

2.6. Uptake assay for radioactive glucose analogues 2-deoxyglucose (DOG) and 3-0-metylglucose (OMG)

The uptake assays were performed as previously described (Angulo et al., 2008). After 20 h of *in vitro* culture, cells were washed gently with 3 mL of incubation buffer (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, pH 7.4, 320 mOsm) and incubated in the same solution for 30 min at 37 °C to allow for equilibrium between the extracellular and intracellular compartments. After completing this period, the cells were pelleted and the uptake assays were conducted for different times (0–90 s) in 400 μL of incubation buffer containing 1–1.2 μCi of 2-deoxy-D-[1,2-$(N)^3$H] glucose (26.2 Ci/mmol; Dupont NEN, Boston, MA, USA). Uptake was stopped by washing the cells with ice-cold incubation buffer containing 0.2 mmol/L $HgCl_2$. Cells were dissolved in 200 μL of lysis buffer (10 mmol/L Tris–HCl pH 8.0 and 0.2% sodium dodecyl sulfate). The incorporated radioactivity was measured by liquid scintillation spectrometry (Castro et al., 2008). The assays were conducted in three biological and experimental replicates at 4 °C. A stop cold solution was added to the radioactive solution to avoid non-specific radioactivity. Three wells were not treated with radioactive or lysis buffer to estimate the average number of total cells using a Neubauer hemocytometer chamber.

2.7. Saturation of glucose analogues 2-deoxyglucose and 3-0-metylglucose

Uptake assays were conducted in 500 μL of incubation buffer containing increasing concentrations from 0 to 80 mM of 2-deoxy-D-glucose or 3-0-methyl glucose and fixed concentration of 1-1.2 uCi 2- [1,2-3H (N)] – deoxy-D-glucose or 1–1.2 uCi of 3-*O*-[methyl-3H]-D-glucose (10Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) as required. Incubation time was 10 s (selected within the range corresponding to the initial speed in the kinetics of incorporation). The assays were conducted in three biological and experimental replicates at 4 °C and radioactivity was measured by liquid scintillation spectroscopy. The Michaelis constant, K_m and V_{max}, were calculated using non-linear regression by Michaelis–Menten equation (hyperbola single rectangular with two parameters).

2.8. Effect of GM-CSF on 2-deoxyglucose (DOG) and 3-0-methylglucose (OMG) uptake in granulosa cells

After 20 h of *in vitro* culture, cells were washed gently with 3 mL of incubation buffer (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, pH 7.4, 320 mOsm) and incubated in the same solution for 1 h at 37 °C to remove cytokines from culture medium. Thereafter, the cells were incubated for 30 min with increasing- concentration of 0–100 nM hGM-CSF 215-GM-010 (R&D System, MN, USA). The uptake assay was conducted for 10 s in three biological and experimental replicates at 4 °C in the presence of 0.5 mM DOG and 0.5 mM OMG.

2.9. Effect of GM-CSF, FSH, IGF-I or their combination on 2-deoxyglucose (DOG) and 3-0-methylglucose (OMG) uptake in granulosa cells

Similar to the procedure described (Section 2.8); cells were washed gently with incubation buffer and incubated in the same solution for 1 h at 37 °C previous to treatment. Thereafter, cells were treated for 30 min with a concentration of 5 mM GM-CSF, 0.055 or 0.22 IU/mL FSH, 10 ng/mL IGF-I or their combinations. The uptake assay was conducted for 10 s in three biological and experimental replicates at 4 °C in the presence of 0.5 mM DOG and 0.5 mM OMG.

2.10. Statistical analysis

Data were transformed to logarithms if they were not normally distributed (Shapiro-Wilk test). One way Analysis of variance (ANOVA) was used to compare the effect of treatments (GM-CSF, FSH, IGF-I or their combination), doses, and replicates on 2-deoxyglucose (DOG) and 3-0-methylglucose (OMG) uptake in granulosa cells using SAS software (SAS, Statistical Analysis System Institute Inc., Cary, NC, USA). Tukey's multiple comparison was used as a post-hoc test when treatment means were significant ($P < 0.05$). Dunnet's test was used to compare the

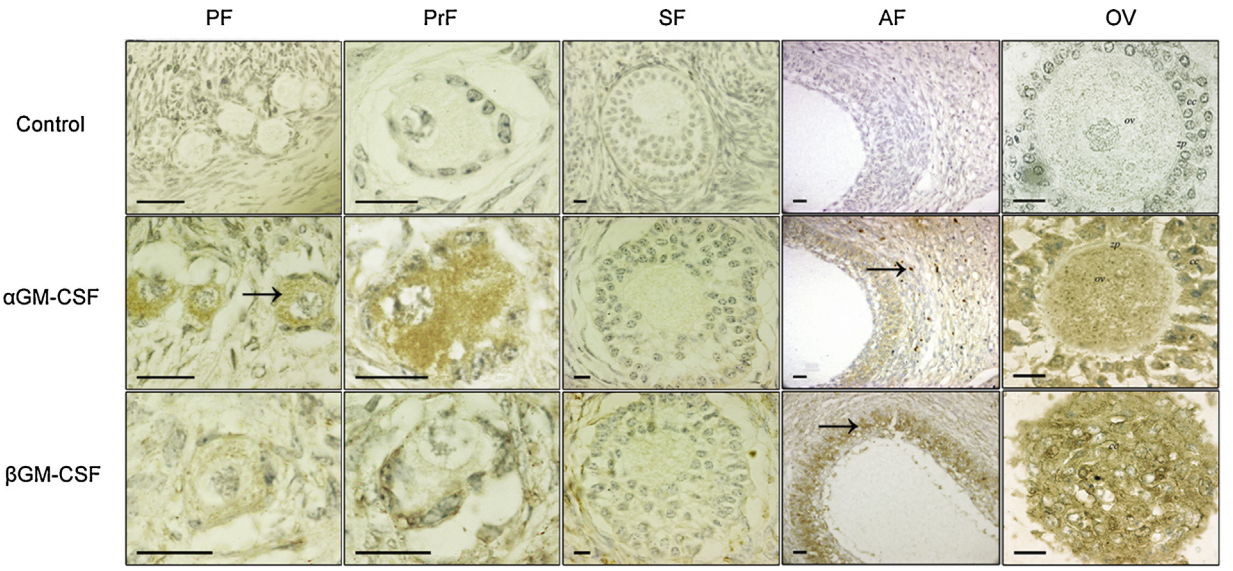

Fig. 1. Immunohistochemical analysis of α- and β- subunits of the GM-CSF receptor during follicular development in cattle. Intense immunoreactivity was observed for the α-subunit of GM-CSF receptor associated to cytoplasm of granulosa cells in primordial and primary follicles (PF and PrF; black arrow). Reactivity for both GM-CSF receptor subunits was weak in granulosa cells of secondary follicles (SF). Punctuate staining associated to the GM-CSF α-subunit was observed in internal and external theca cells of antral follicles (AF; black arrow). In contrast, staining associated to the GM-CSF β-subunit was detected in granulosa cells of AF (black arrow). Immunoreactivity for both GM-CSF subunits was observed in oocytes (OV) and cumulus cells. Negative control incubated with non-immune serum instead of primary antibody. The staining of slides for different follicles and antibodies was compared simultaneously. Scale bars = 50 μm (PF and PrF), 10 μm (SF), 20 μm (AF and OV).

treatments with the control group. Hyperbolic regression analysis, exponential curve adjustment and lineal regression for the radioactive glucose analogues 2-deoxyglucose (DOG) and 3-0-metylglucose (OMG) uptake assay were analyzed using Sigma Plot 9.0. (Jandel).

3. Results

3.1. Expression of the α- and β-subunit genes of GM-CSF receptor from primordial to antral stage of follicular development

Immunoreactivity for α- and β-subunits of the GM-CSF receptor was detected in primordial and primary follicles. The α-subunit was detected in the cytoplasm of oocytes and granulosa cells (Fig. 1). Reactivity for both GM-CSF subunits was observed in granulosa cells of secondary follicles. Punctuate staining associated with the GM-CSF α-subunit was observed in internal and external theca cells of antral follicles. In contrast, widespread staining associated with the GM-CSF β-subunit was detected in granulosa cells in the majority of the antral follicles.

3.2. Expression of the α- and β-subunit genes of GM-CSF, 3β-HSD, FSHR and GLUTs transporters in isolated granulosa cells from antral follicles

Immunofluorescence analyses detected expression of both GM-CSF α- and β- subunits and FSHR associated with the plasma membrane in granulosa cells isolated from antral follicles (<4, 4–8 and >8 mm) (Fig. 2). Moreover, both subunits of the GM-CSF receptor were co-localized with 3β-HSD in granulosa cells. Immunoreactivity for both GM-CSF subunits and FSHR were observed by western blots and immunofluorescence in granulosa cells from <4, 4–8 and >8 mm follicles in diameter (Fig. 3). Western blot analyses of granulosa cells from all follicle categories detected immunoreactive bands for GM-CSF α- and β-subunits and FSHR (∼82, ∼115 and ∼60 kDa, respectively, Fig. 3A). A representative, positive immunofluorescence to GM-CSF α- and β-subunits in granulosa cells isolated from 4 to 8 mm follicles and co-localized with FSHR is displayed in Fig. 3B. The expression of GLUT1, GLUT2, GLUT3 and GLUT4 genes was detected by western blot and immunofluorescence analyses in granulosa cells isolated from <4, 4–8 and >8 mm follicles in diameter. Immunoreactive bands and immunofluorescence were observed for GLUT 1, GLUT2, GLUT3 and GLUT4 (∼48, ∼55, ∼53 and ∼57 kDa, respectively) in granulosa cells isolated from all follicle categories (Fig. 4A and B). A punctuate pattern of immunostaining associated to GLUTs gene expression was observed in the cytoplasm or plasma membrane of granulosa cells (Fig. 4B).

3.3. Uptake assay for radioactive glucose analogues 2-deoxyglucose (DOG) and 3-0-metylglucose (OMG)

The uptake kinetic assay of DOG was conducted at 4 °C for an incubation time of 90 s in a primary culture of granulosa cells isolated from 4 to 8 mm antral follicles. Deoxyglucose is a substrate of facilitative glucose transporters, and the transported deoxyglucose accumulates intracellularly in cell as deoxyglucose-6-phosphate (Carruthers, 1990). Discrimination between transport and accumulation is usually accomplished experimentally by carrying out very short uptake assays. The analysis suggested that the uptake was linear for the first 20 s of incuba-

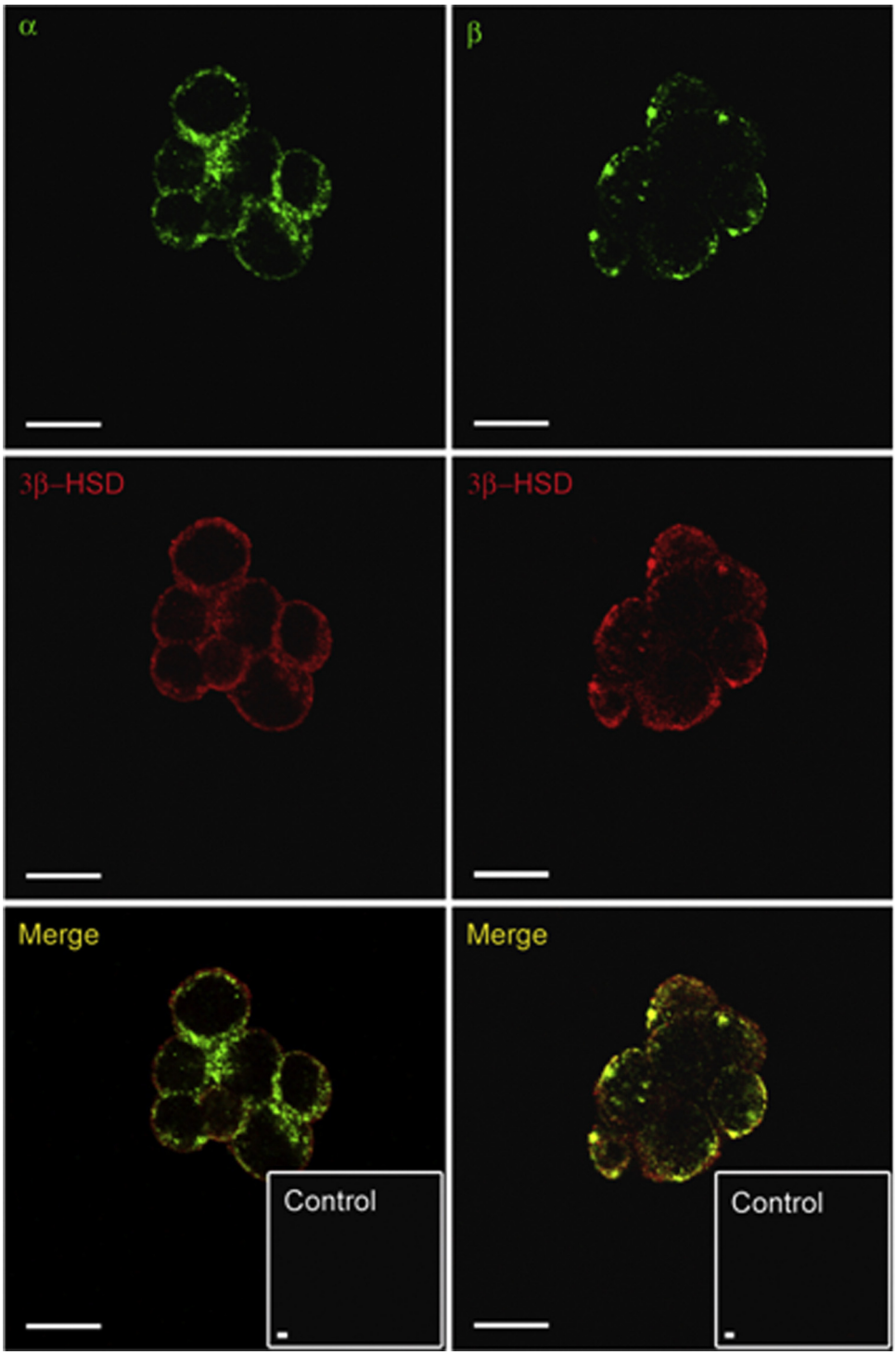

Fig. 2. Immunolocalization analysis of α- and β- subunits of the GM-CSF receptor and 3β-HSD in granulosa cells of cattle. Immunofluorescence for α- and β- subunits of the GM-CSF receptor (Green) and 3β-HSD enzyme (Red) were associated to the plasma cell membrane. α- and β- subunits of the GM-CSF receptor and 3β-HSD co-localized (Yellow) in granulosa cells. Negative control incubated with non-immune horse serum instead of primary antibody. The staining of slides for different antibodies was compared simultaneously. Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

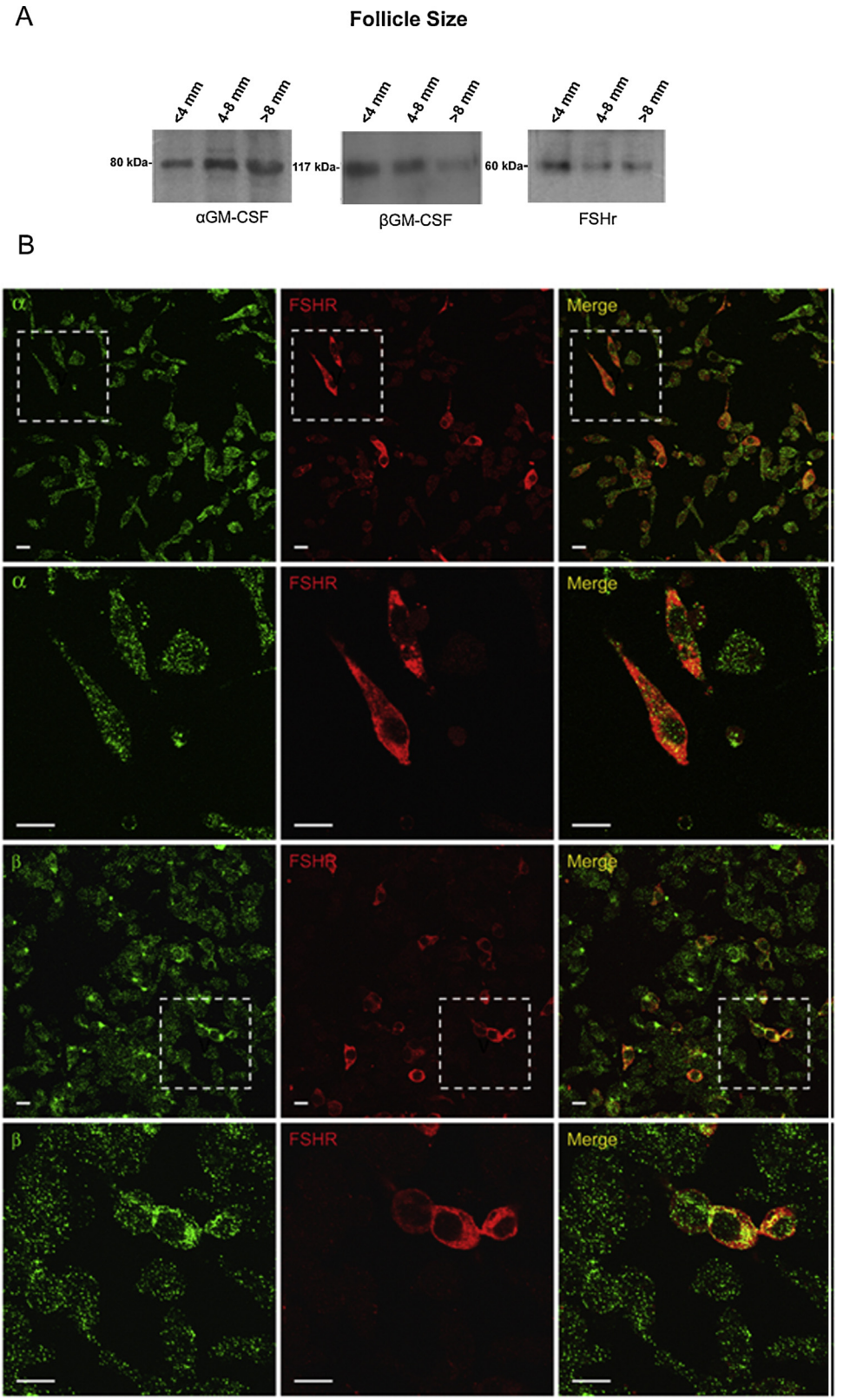

Fig. 3. Expression of α- and β- subunit genes of the GM-CSF receptor and FSHR in granulosa cells isolated from follicles of different sizes in cattle. (A) Western blot analyses detected immunoreactive bands for α- and β-subunits of GM-CSF receptor and for FSH receptor (~80, ~117 and ~60 kDa, respectively) in granulosa cells at all follicular sizes analyzed. (B) Immunofluoresence associated to GM-CSF α- and β-subunits (Green) and FSH r (Red) co-localized (Orange)

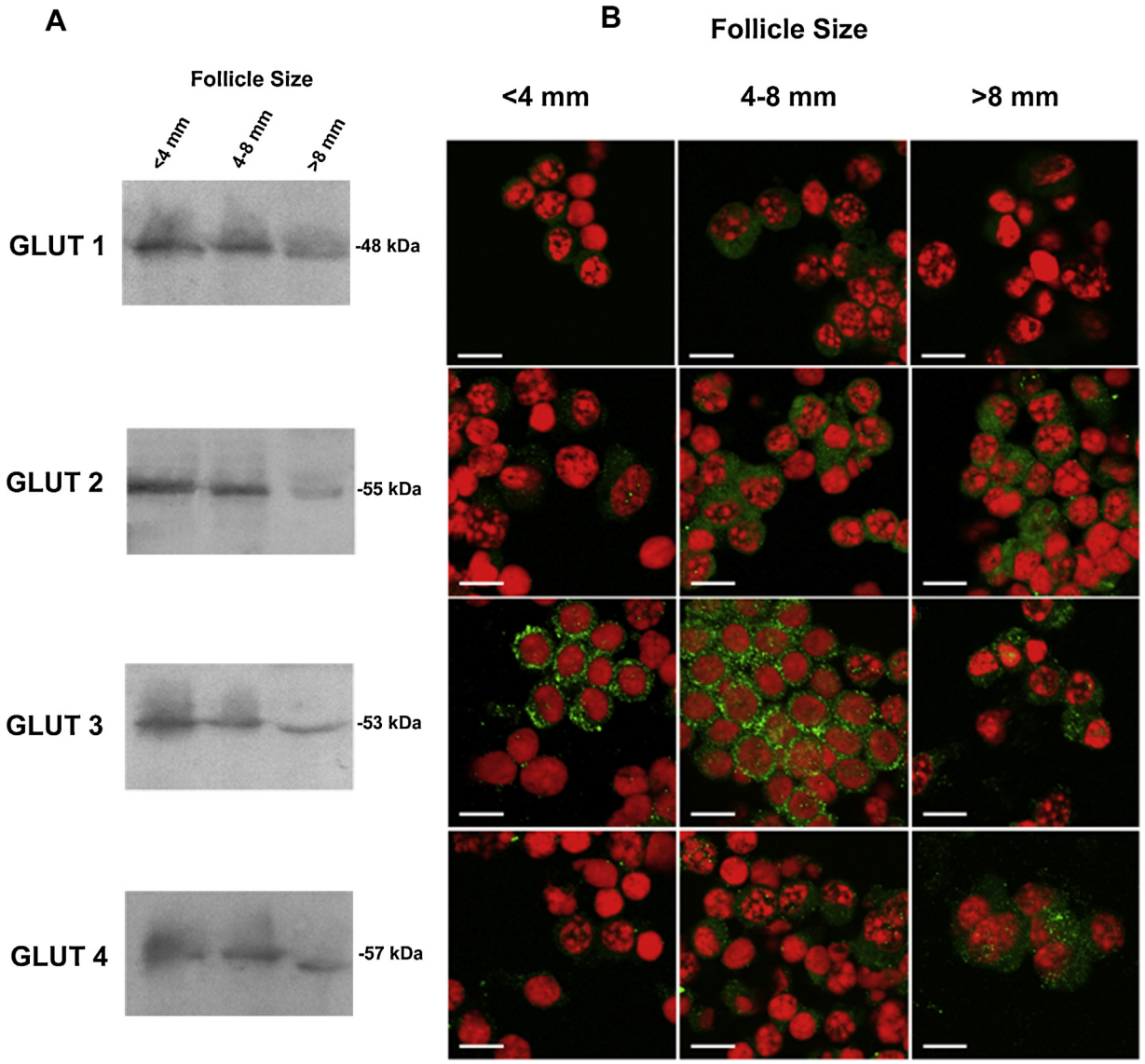

Fig. 4. Expression of GLUT1, GLUT2, GLUT3, and GLUT4 gene in granulosa cells during follicular development in cattle. (A) Western blot analyses detected immunoreactive bands with variable intensities for GLUT1, GLUT2, GLUT3, and GLUT4 (~48, ~55, ~53 and ~57 kDa, respectively) in granulosa cells at all follicular sizes analyzed. (B) Immunofluorescence associated to GLUT1, GLUT2, GLUT3 and GLUT4 (Green) was observed in all granulosa cells isolated from antral follicles. Strongest immunoreactivity was detected for GLUT3 in granulosa cells from follicles of sizes <4 mm and 4–8 mm. Similarly, signal for GLUT2 was strong in granulosa cells isolated from follicles of sizes 4–8 mm and >8 mm. Cell nuclei were stained with propidium iodine (Red). Negative control incubated with non-immune serum instead of primary antibody. The staining of slides for different follicles and antibodies was compared simultaneously. Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tion and, thereafter plateaued (Fig. 5A). Thus, an incubation time of 10 s was considered in the following assays to obtain an initial transport velocity. The initial velocity for DOG transport was 42.5 ± 11.1 nmol/10^6 cells/min. As it was expected for hexose transporters, the uptake saturation curve under increasing concentrations of DOG behaves as a hyperbole (Fig. 5B). This curve generated the kinetics parameters of Vmax = 62.8 ± 14.7 nmoles/10^6 cells/min and Km = 1.9 ± 1.0 mM. Although, the results obtained with DOG confirmed that granulosa cells display functional hexoses transporters, DOG is metabolized and phosphorylated in the cells; therefore, it makes it difficult to study differences between the intracellular uptake and storage processes. Because of a close relationship between the uptake and the phosphorylation process exists; uptake analysis of OMG, a non-metabolizable glucose analogue

in granulosa cells isolated from antral follicles. Negative control incubated with non-immune serum instead of primary antibody. The staining of slides for different antibodies was compared simultaneously. Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

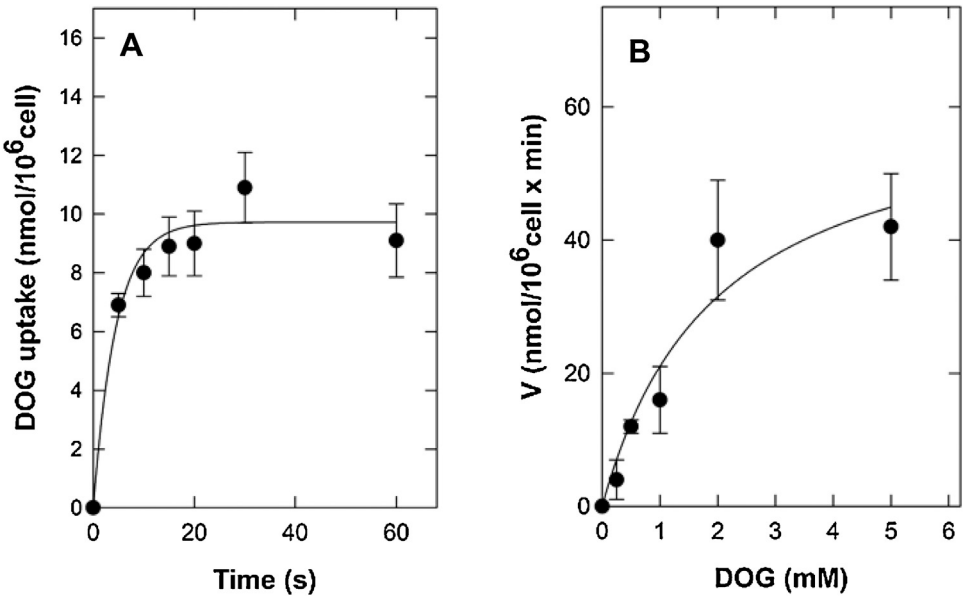

Fig. 5. Kinetics for the uptake of deoxyglucose by primary granulosa cell culture from antral follicles of 4–8 mm in cattle. (A) Time course of the uptake of 0.5 mM deoxyglucose (DOG). (B) Dose-response of the transport of DOG using 10-s uptake assays. Single rectangular hyperbolas (A and B) were fitted to the data using nonlinear regression ($R = 0.985$). The assay was conducted in triplicate and the mean ± SD of each data correspond to five independent trials.

that is incorporated into the cell down a concentration gradient, was included in the uptake assays. The values obtained were similar to those observed for 2-DOG (Robertson et al., 2001; Zambrano et al., 2010). Thus, it was confirmed the presence of facilitative hexose transporters and also the optimum conditions for conducting the subsequent kinetic studies.

3.4. Effect of GM-CSF on 2-deoxyglucose (DOG) and 3-0-methylglucose (OMG) uptake in granulosa cells

GM-CSF induced a significant increase in 2-DOG and 3-OMG uptake with respect to the control group in granulosa cells (Fig. 6A and B). GM-CSF at a concentration of 5 nM increased 2-DOG uptake up to 30% (Fig. 6A), whereas a greater concentration of the cytokine did not induce further increases. GM-CSF at a concentration of 5 nM induced an increased uptake of 3-OMG in granulosa cells to 35.6 ± 11.2% greater than the control (Fig. 6B).

3.5. Effect of GM-CSF, FSH, IGF-I or their combination on 2-deoxyglucose (DOG) and 3-0-methylglucose (OMG) uptake in granulosa cells

The effect of GM-CSF, FSH, IGF-I or the combinations of these hormones on glucose analogues uptake in granulosa cells are depicted in Fig. 7. GM-CSF, FSH and IGF-I used alone or in combination increased DOG or OMG uptake in granulosa cells. FSH and IGF-I significantly increased glucose uptake up to 60.1 ± 4.3% and 47.2 ± 14. 7% greater than the glucose uptake in the control group, respectively (Fig. 7A and B). No additive effect was observed when cells were treated with a combination of GM-CSF with FSH (Fig. 7A). Moreover, GM-CSF plus FSH induce the same DOG uptake compare to GM-CSF alone (Fig. 7A). Supplementation of GM-CSF plus IGF-I enhanced glucose uptake to 61.0 ± 22.1% above the control, and this effect was significantly different compared with the use of GM-CSF alone (Fig. 7B). Combination of GM-CSF, IGF-I and FSH resulted in similar ($P > 0.05$) DOG uptake compared to GM-CSF alone and combination of IGF-I and FSH (Fig. 7C). Regarding the OMG assay, FSH had a significant effect on glucose analogue uptake from concentration of 0.026 IU/mL (increase in 74.0 ± 10.0% greater than the control) to a concentration of 0.22 IU/mL (increase in 124.9 ± 15.0% greater than the control; Fig. 7D). GM-CSF, FSH and IGF-I alone increased ($P < 0.05$) OMG uptake compared to the control group (Fig. 7E). Unlike the results observed with DOG, the combination of IGF-I and FSH resulted in an increase in OMG uptake up to 150.7 ± 22.3% of the control and this value was greater ($P < 0.05$) compared with the effect of IGF-I alone. It should be noted that the concentration of FSH used in these experiments was four times greater than that used with DOG; therefore, the observed synergy may be given by the concentration factor. Although, the combination of IGF-I, FSH and GM-CSF significantly increased OMG uptake up to 88.7 ± 12.9% than that of the control group, the increase was still less than with the combination of IGF-I and FSH.

4. Discussion

The expression of both subunit genes of the GM-CSF receptor was evaluated with the aim to reveal its spatial association to FSHR and its temporal gene expression with various GLUTs at different stages of ovarian folliculogenesis in cattle. In the present study, it was found that low affinity GM-CSF α-subunit was specifically located in granulosa cells of primordial and primary follicles as well as in the theca cells of antral follicles. In contrast, immunoreactivity for high affinity GM-CSF β-subunit was mainly

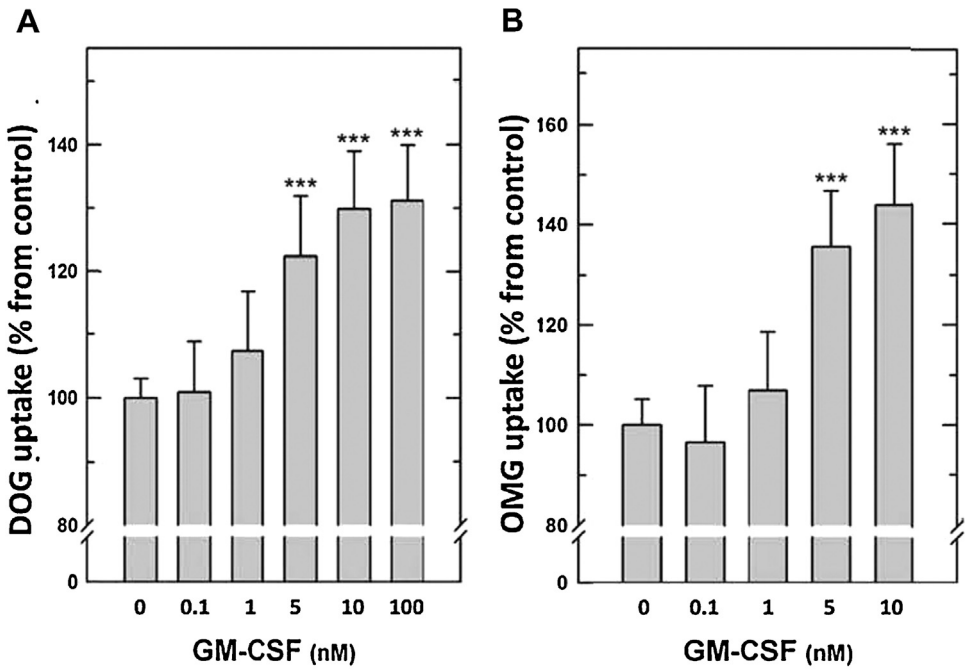

Fig. 6. Effect of GM-CSF on DOG and OMG uptake in granulosa cells of cattle. Granulosa cells were cultured for 20 h and pre-incubated in incubation 3 mL buffer for 1 h before treatment. The cells were then incubated for a period of 30 min with increasing concentration of GM-CSF (0–100 nM). The assay was performed at 4 °C for 10 s in presence of 2-DOG (0.5 mM) (A) and 3-OMG (0.5 mM) (B). Each assay was performed in triplicate and bars represent the mean ± SD of five independent trials. ***$P < 0.001$ significance between treatment versus control group.

detected in granulosa cells of antral follicles. These gene expression patterns of α- and β-subunits of the GM-CSF receptor during ovarian folliculogenesis in cattle corresponds with previous analyses in the human ovary, where GM-CSF receptor subunits were immuno-detected in theca externa and granulosa cells of large follicles (Zhao et al., 1995). The expression of subunit genes of GM-CSF receptor in cattle follicles supports the rationale that GM-CSF may have a potential role during folliculogenesis. However, in cattle, this potential role may be limited to granulosa and cumulus cells, because it was previously reported (Peralta et al., 2013) that GM-CSF stimulated cumulus cell gene expansion in a dose response manner without altering blastocysts production in an *in vitro* fertilization system.

Previous studies have attempted to explore the potential role of GM-CSF in follicular development and have suggested that the mechanisms underlying this effect may be difficult to reveal. In one study, authors indicated that GM-CSF may exert an indirect effect in granulosa and cumulus cells mediated by steroidogenesis-regulating secretion of local macrophage populations residing in the theca (Gilchrist et al., 2000). This effect may induce differentiation and maturation of follicular cells and may alternatively be mediated through an intermediate cell T-I population responsible for regulation of ovarian steroidogenesis, follicular growth, follicular atresia and ovulation (Gilchrist et al., 2000; Wang et al., 2005; Makinoda et al., 2008). In the present study, the colocalization of α- and β-subunits of GM-CSF receptor with 3β-HSD and FSHR along with the expression of the GLUTs gene in granulosa cells of cattle could suggest a potential role of this cytokine during follicular development. 3β-HSD is an enzyme that catalyzes several steroidogenic reactions including synthesis of progesterone from pregnenolone and androstenedione from dehydroepiandrosterone (Payne et al., 1995). Moreover, the 3β-HSD gene is expressed in granulosa cells and has an important role as regulator of progesterone biosynthesis in differentiating estrogenic granulosa cells (Conley et al., 1995; Sahmi et al., 2004). The FSHR gene is expressed in granulosa cells and mediates the effect of FSH in the synthesis of aromatase enzyme and subsequent production of estrogens and progesterone (George et al., 2011; Ryding et al., 2013).

Results of the present study demonstrated that the glucose transporter genes, GLUT1, GLUT2, GLUT3 and GLUT4, are expressed in cattle granulosa cells from follicles that are <4–>8 mm in size. Glucose transporter genes are expressed according to tissue type and exhibit different capacities according to metabolic conditions. While the GLUT1 gene is expressed mainly in the brain and erythrocytes, the GLUT2 gene is expressed primarily in pancreatic β-cells where there is participation in the glucose-sensing mechanism (Wood and Trayhurn, 2003). The GLUT3 is a high-affinity glucose transporter in which the gene is expressed mainly in tissues with great demand of glucose, particularly the brain (Wood and Trayhurn, 2003). GLUT4 is an insulin-responsive glucose transporter found in heart, skeleton and adipose tissue (Wood and Trayhurn, 2003). Previous studies (Nishimoto et al., 2006) have reported the presence of mRNA of GLUT 1, 3 and 4 in cattle granulosa cells, and have suggested that GLUT1 and GLUT3 functions as a major glucose transporter whereas,

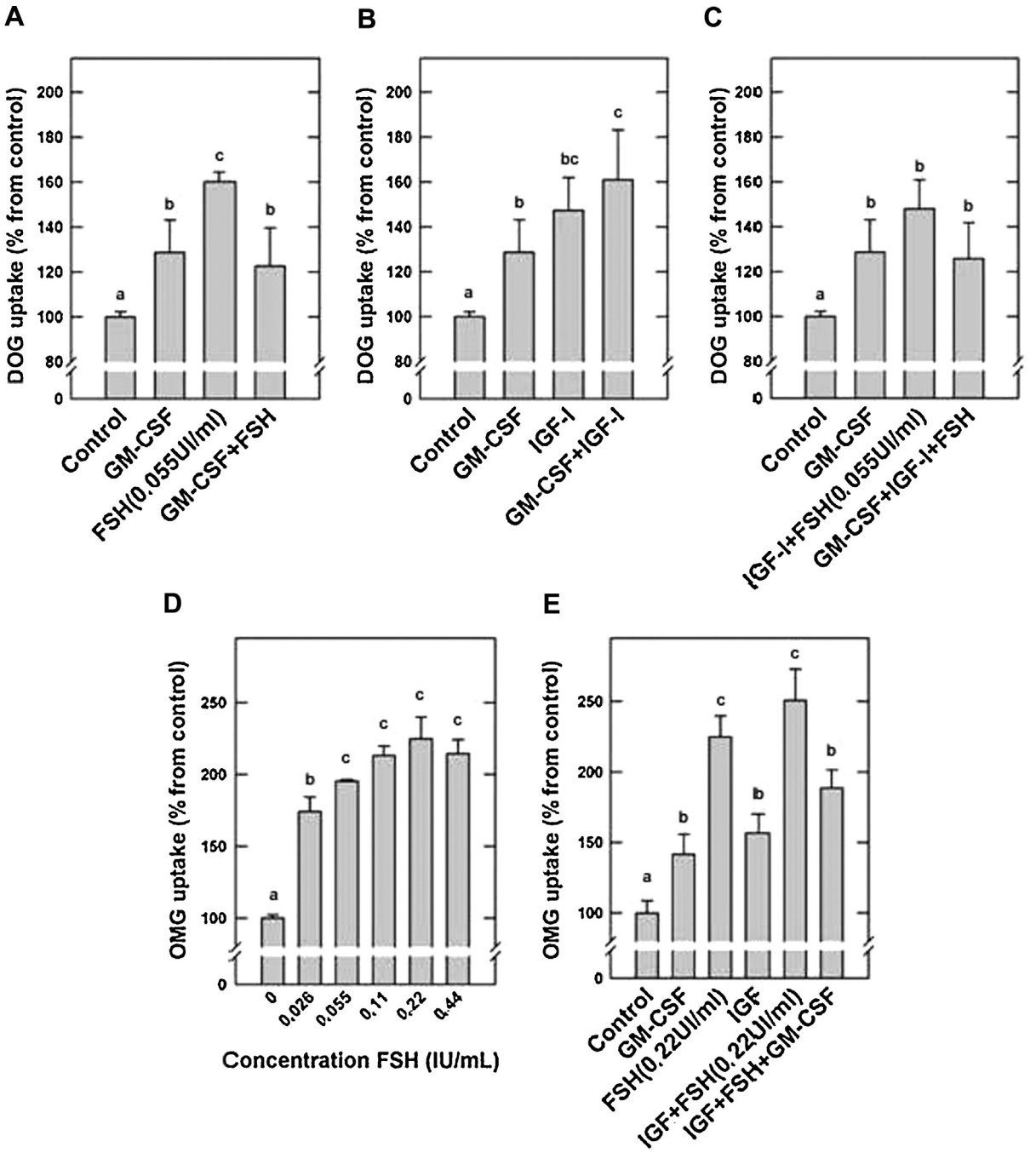

Fig. 7. Effect of GM-CSF, FSH and IGF-I on DOG and OMG uptake in granulosa cells of cattle. Granulosa cells were cultured for 20 h and then pre-incubated in 3 mL of incubation buffer for 1 h before treatment. Then cells were incubated for a period of 30 min with different stimuli. The uptake assay was performed at 4 °C in presence of 2-DOG (0.5 mM) (A–C) or 3-OMG (0.5 mM) (D, E) and was measured for 10 s. The concentration of GM-CSF and FSH were 5 nM and 0.055 IU/mL respectively in DOG assay (A–C). FSH concentration was 0.22 IU/mL in OMG assay (D, E). IGF-I was used at a concentration of 10 ng/mL. Bars represent the mean ± SD from three replicates. [a,b,c] Letters with different superscript differ among treatments groups ($P < 0.05$) using the *post hoc* Tukey 's test.

GLUT4 has a supporting role in function of the follicle tissues. Moreover, up-regulation of GLUT1 and GLUT3 gene expression after glucose deprivation represents a compensatory mechanism for glucose uptake that maintains energy supply to follicular cells (Nishimoto et al., 2006). In the present study, the GLUT1, GLUT2, GLUT3 and GLUT4 immunostaining in cattle granulosa cells, suggests participation of glucose transporter gene expression during ovarian follicular development. Uptake kinetic assays confirmed not only presence and functionality of facil-

itative hexose transporters in cattle granulosa cells but also the bioactivity of the GM-CSF and FSH receptors. Uptake kinetic curves of metabolizable glucose analogue DOG and non-metabolizable glucose analogue OMG were similar indicating that glucose was transported and phosphorylated intracellularly. A role for GM-CSF in regulating granulosa cell metabolic activity was determined, specifically by promoting DOG and OMG uptake increase in ~30%, following treatment with 5 nM GM-CSF. Previous studies have reported increased uptake of DOG in *Xenopus laevis* oocytes, through phosphorylation-independent GM-CSF effect on endogenous glucose transporters (Ding et al., 1994). Similar effects of GM-CSF were seen in HL-60 cells, normal human neutrophils and melanoma cells, which endogenously express GM-CSF receptor in the absence of high affinity β-subunit (Spielholz et al., 1995). In this context, it has been reported that GM-CSF modulates several metabolic signaling pathways, promotes glucose uptake through direct activation of the GM-CSF receptor, and interacts with glucose transporters. GM-CSF induces translocation of GLUT1 to the plasma membrane and subsequent activation of PI 3-kinase/PKB pathway in human embryonic kidney cells (Zambrano et al., 2010). The intrinsic efficiency of GLUT 1 is also enhanced after GM-CSF increase the Km value without changing the V max (Fiorentini et al., 2001). GM-CSF may also activate glucose uptake in murine macrophages by increasing GLUT 3 affinity for glucose (Ahmed et al., 1997). Moreover, GLUT 3 and GLUT 5 have been co-localized with GM-CSF receptor in midpiece and tail of bull spermatozoa suggesting that a physical interaction between these proteins is required for hexose uptake (Vilanova et al., 2003). Although future studies are required for clarification, previous and present results herein suggest that GM-CSF regulates glucose transport in cattle granulosa cells during folliculogenesis.

Treatment with GM-CSF, FSH or IGF-I alone increased DOG or OMG uptake in granulosa cells. When cells were treated with combination of these factors there was not an additive effect. Unexpectedly a combination of GM-CSF and FSH decreased DOG uptake. IGF-I and FSH treatment on granulosa cells have been associated with cell differentiation including increased cell number and estradiol production (Gutierrez et al., 1997). IGF-I enhanced glucose oxidation in mature granulosa cells of pigs (Weber and La Barbera, 1988). FSH induces direct control over carbohydrate metabolism in granulosa cells controlling lactate accumulation (Hillier et al., 1985; Roberts et al., 2004; Sutton et al., 2003). The positive effect of FSH on granulosa cells glucose uptake is significant during all stages of follicular development (Boland et al., 1994). In the present study, FSH exerted a potent metabolic effect on cattle granulosa cells by increasing DOG and OMG uptake. In contrast, it has been reported that IGF-I functions in an autocrine manner on granulosa cells, and in a paracrine manner on oocytes to increase GLUT 1 gene expression and subsequently induces glucose transport (Zhou et al., 2000). While studies have showed an interaction between IGF-I and GLUT1 gene expression (Zhou et al., 2000), other reports have indicated that FSH increases glucose uptake by translocation of GLUT4 to the granulosa cell membrane (Roberts et al., 2004).

In conclusion, the expression pattern of both α- and β-subunits of the GM-CSF receptor and its spatial association with 3β-HSD and FSHR and temporal expression of the GLUTs gene in granulosa cells during folliculogenesis in cattle are reported in the present study. GM-CSF, FSH and IGF-I promote glucose uptake in granulosa cells and; however, the reduced effect of combined GM-CSF and FSH suggests an interaction of these factors for glucose transport. The spatial association of GM-CSF receptor with 3β-HSD and FSHR as well as the GLUTs gene expression in granulosa cells results in an interesting cellular model to study the effect of this cytokine on the functionality of hexose transporters during folliculogenesis in cattle.

Conflict of interest

None of the authors have any conflict of interest to declare.

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