Characterization of chicken Sertoli cells in vitro

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ABSTRACT In the testis, Sertoli cells play a key physiological role in that they support, nourish, and protect germ cells. Because of the importance of Sertoli cells, several laboratories have established a culture system of Sertoli cells. These cultures have been well developed in mammalian species, but to our knowledge no purified avian Sertoli cells culture has been described. The aim of this study was to isolate avian Sertoli cells and to investigate their function using a chicken model in an in vitro test system. Immature chicken Sertoli cells in culture present morphology similar to that of mammalian cells and conserve expression of the specific Sertoli marker, anti-Müllerian hormone. Furthermore, in contrast to mammals, they express the 3β-hydroxysteroid dehydrogenase enzyme. Stimulation of Sertoli cells with ovine follicle-stimulating hormone rapidly activates the 3 main downstream signaling pathways of the follicle-stimulating hormone receptor: cyclic adenosine monophosphate/protein kinase A, phosphatidylinositol 3-kinase/Akt, and mitogen-activated protein kinase pathways. In vitro, Sertoli cells are able to secrete lactate and inhibit and have conserved the phagocytosis property. Finally, avian Sertoli cells present 3 interesting characteristics: they actively proliferate in vitro, can be passaged several times, and are suitable for freezing in nitrogen. A direct consequence of these properties is to use this cell culture test system as an alternative method to bird reprotoxicity studies.

Key words: chicken, testis, Sertoli cell, in vitro culture

INTRODUCTION

Spermatogenesis is a process involving a multitude of cellular interactions and involves 2 important somatic cell types: Sertoli and Leydig cells. Leydig cells produce testosterone and Sertoli cells support germ cells in the seminiferous tubules and provide them with growth factors and energy (de Reviers et al., 1980). The secretion of all of these factors contributes to a microenvironment that can regulate the balance between self renewal of spermatogonia and their differentiation in spermatozoa. These cellular functions are modulated by pituitary gonadotropins such as luteinizing hormone in regulating mainly Leydig cells and follicle-stimulating hormone (FSH) in modulating Sertoli cells (Tanaka and Yasuda, 1980; González-Morán, 1997).

Several laboratories have developed a culture system of testicular cells based mainly on rat species (Steinberger et al., 1975; Le Magueresse et al., 1986). The strength of these culture systems is to analyze the development and activity of somatic and germ cells in a controlled culture medium. Hence, in the last decade, in vitro studies have demonstrated the role of molecules present in the environment and their possible effects on male reproductive functions in vivo (Steinberger and Klinefelter, 1993; Habert et al., 2009). These molecules are of natural origin (phytoestrogens) or chemical compounds with mainly antiandrogenic or estrogenic activity that may disrupt testicular development. Transfer of this system to other mammalian models such as mice and humans have shown specificities related to the species (Habert et al., 2009). In avian species, only chicken Leydig cell culture (Opalka et al., 2004) and culture of dispersed chicken testicular cells (Glenn et al., 1981; Rombauts et al., 1995; Mi et al., 2004) have been reported. To our knowledge, no purified avian Sertoli cell culture has been described.

Nonetheless, at the opposite of mammals, chicken testis presents some particularities such as temperature of 41 to 43°C because of the location of testes in the deep body and absence of the pampiniform structure (Lake, 1971). Spermatozoa are produced in a shorter period compared with other vertebrates (the duration of the spermatogenic process is 14 d in chickens, 35 d in mice, and 64 d in humans; Heller and Clermont, 1963; Brillard and de Reviers, 1981; Adler, 1996), and the testicular activity is strongly controlled by the photoperiod, suggesting a relative plasticity and rapidity to...
produce sperm across seasons (de Reviers et al., 1980; Brillard, 1986). In addition, in chickens the testicular growth starts slowly until 5 wk of age (1 mg/d) and then progresses very quickly between 6 and 20 wk (87 mg/d; Reviers, 1971). In both mammals and birds, several studies have associated the testis size and the production and quality of germ cells with the number and function of Sertoli cells (de Reviers et al., 1980; Orth et al., 1988; Rosenstrach et al., 1994). Indeed, each Sertoli cell supports a limited number of germ cells and they support and nourish germ cells during their development. Sertoli cells secrete several proteins such as hormones [e.g., anti-Müllerian hormone (AMH), inhibin, insulin-like growth factor 1 (IGF-1)], transport proteins, lactate, or participate in the production of polyunsaturated fatty acids involved in the composition of the cell membrane (including germ cells membrane; Retterstøl et al., 2001).

The aim of this study was to isolate avian Sertoli cells and investigate their functions using chicken species. Several expected characteristics of Sertoli cells (gonadotropin sensitivity, lactate and inhibin secretions, and phagocytosis) were evaluated by using an in vitro test system.

**MATERIALS AND METHODS**

**Cell Culture**

Reagents used for cell culture preparation were described previously (Froment et al., 2007). Recombinant human IGF-1 was a gift from P. Swift (Ciba-Geigy, Saint Aubin, Switzerland) and ovine FSH (oFSH) was purchased from Sigma (St. Louis, MO). Sertoli cells preparations were based on rodent procedures as described in Mather and Phillips (1984) and Odet et al. (2006), with enzymatic treatments adapted for chicken species.

Sertoli cells were obtained from 6- or 8-wk-old (immature) laying breed chickens (ISA Brown, egg layer type; Institut de Selection Animale, Saint Brieuc, France); the exception was Sertoli cells purified from testes with a weight of 500 mg and 1,000 mg, which were obtained from 8.5- and 10-wk-old birds, respectively. Interestingly, the same protocol has been tested to successfully prepare Sertoli cells from immature or adult animals (1-, 6-, 8-, 10-, and 40-wk-old chickens; P. Froment and E. Guibert, UMR 6175, INRA, CNRS Université de Tours Haras Nationaux, Physiologie de la Reproduction et des Comportements, Nouzilly, France; unpublished data). All the animals were housed in individual battery cages under a 14-h light:10-h dark photoperiod, and feed and water were provided ad libitum.

For each culture, 4 chickens were killed by cervical dislocation. Testes were immediately recovered and pooled in cold culture medium. For cell preparation, HEPES-buffered F12/ Dulbecco’s modified eagle’s medium (DMEM; Sigma) supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma) was used. Testes were decapsulated, slightly minced, and incubated for 15 min at 37°C with DNase (20 µg/mL) and type Ia collagenase (0.4 mg/mL; Sigma). Cells were centrifuged to remove collagenase and were allowed to sediment by gravity to separate seminiferous tubules, in the pellet, and Leydig cells, in the supernatant. The pelletted seminiferous tubules were resuspended and digested by 2 collagenase baths (0.6 mg/mL and 0.8 mg/mL for 15 min at 37°C each digestion) added with Dnase (20 µg/mL) and followed by 0.1% hyaluronidase treatment (Sigma) for 10 min at 37°C to reduce peritubular cell contamination. Contamination of Sertoli cell preparations with few germ cells (<10%) was no longer present after 2 d in culture (data not shown) and the percentage of peritubular myoid cells, evaluated by alkaline phosphatase staining, was close to 8% of the total cell population (8 ± 0.9%; n = 3).

The cells were counted and seeded to appropriate density in HEPES-buffered F12/DMEM supplemented with antibiotics and 5% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO2 in air. Ninety-six-well plates were used for methyithiazolyldiphenyl-tetrazolium bromide (MTT) assay (5 × 103 cells/well); 6-well plates were used for mRNA, proteins, hormone secretions analysis (5 × 105 cells/well); and chamber preparations were based on rodent procedures as described in figure captions and in Results. At the end of treatments, media were collected and stored at −20°C until determination of cyclic adenosine monophosphate (cAMP), inhibin, and lactate concentrations. Cells were washed with PBS and then frozen at −80°C.

Mouse Sertoli cells, used as control in Figure 1, were prepared from 20-d-old mice as described in Froment et al. (2007). The quail muscle cell line QM7 (Antin and Ordahl, 1991) was cultivated in HEPES-buffered F12/DMEM supplemented with antibiotics and 10% FCS at 37°C in a humidified atmosphere of 5% CO2 in air.

**3-β-Hydroxysteroid Dehydrogenase and Phosphatase Alkaline Activities**

The presence of the 3-β-hydroxysteroid dehydrogenase (3β-HSD) activity was evaluated by incubating a
Figure 1. A) Representative microscopic fields of (1, 2) chicken Sertoli cells and (3) mouse Sertoli cells cultured in vitro. (4) Cholesterol accumulation localized around the nucleus was visualized after filipin staining. B) Expression of chicken anti-Müllerian hormone (AMH) was analyzed by reverse-transcription (RT) PCR in purified chicken Sertoli cells. C) (1) Testicular sections immunostained for AMH in seminiferous tubules of a 4-wk-old chicken. (2) Representative microscopic field of AMH staining localized in cytoplasm of chicken Sertoli cells cultured in vitro. D) Representative microscopic fields of alkaline phosphatase activity. Alkaline phosphatase activity was determined by hydrolysis and reduction of nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate producing formazan and an indigo dye that together form a black-purple precipitate in (1) testicular cell (containing peritubular cells; see arrows) but not in (2) Sertoli cell preparation. E) Expression of chicken 3β-hydroxysteroid dehydrogenase (3β-HSD) was analyzed by RT-PCR in purified chicken Leydig and Sertoli cells. F) Representative microscopic fields of the 3β-HSD activity. The presence of the 33-HSD activity was evaluated by a colorimetric technique based on the dehydrogenase activity of the enzyme as described previously by Chiappe et al. (2002). (1) Chicken Leydig cells and (2) Sertoli cells have shown 3β-HSD activity. Nuclei were counterstained with 4,6-diamidino-2-phenylindole. G) Level of the 3β-HSD protein as determined by Western blot in chicken Sertoli cells stimulated or not by 100 ng/mL of ovine follicle-stimulating hormone (FSH) for 48 h. Vinculin served as a loading control. Color version available in the online PDF.
precursor, dehydroepiandrosterone, in the culture medium that once metabolized 3β-HSD precipitates. This colorimetric technique is based on the dehydrogenase activity of the enzyme as described previously by Chiappe et al. (2002). Negative control was performed by omitting the precursor, dehydroepiandrosterone (data not shown).

Alkaline phosphatase is a marker for myoid cells (Palombi and Di Carlo, 1988). Alkaline phosphatase activity can be detected by staining with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as described in Sonne et al. (2009). Nuclei were stained with 4,6-diamidino-2-phenylindole.

**Phagocytosis Assay**

Uptake of red fluorescent-labeled latex beads of 1 µm (Sigma) was used as a measurement of general phagocytic activity of Sertoli cells. Briefly, Sertoli cells were seeded in chamber slides at 5 × 10^4 cells/well. The following day, Sertoli cells were washed 2 times in serum-free media, and fluorescent latex beads, 1 × 10^6 in 100 µL of culture medium, were added to the well. Because the kinetics of phagocytosis in Sertoli cells are relatively slow (Filippini et al., 1989), cells were incubated for 6 h to ensure internalization of the beads. Then, Sertoli cells were washed 3 times with HEPES-buffered F12/DMEM and fixed for 15 min with paraformaldehyde 4%. The nucleus was stained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and then cells were counted under a fluorescence microscope. One hundred cells were counted in each test, and the results were presented as the mean value of 3 experiments.

**Viability and Cell Proliferation**

Cell viability was estimated by an MTT (Sigma) colorimetric assay based on the conversion of MTT to MTT-formazan product by mitochondrial dehydrogenases from living cells. Four hours before the measurement, the medium was replaced by a medium without red pheno and containing 10% MTT solution. Then, an MTT solution containing isopropanol and 0.1 N HCl was added. The absorbance of the purple MTT formazan was measured spectrophotometrically using a microplate reader. The amount of MTT formazan produced was proportional to the number of viable cells. Results correspond to the mean of 3 independent experiments performed in triplicate.

For BrdU incorporation, the cultured cells were labeled for 24 h with 10 µM BrdU (Sigma). The cells were then fixed for 10 min in 4% paraformaldehyde-PBS. The BrdU-positive cells were identified by indirect immunofluorescence as described by Migliorini et al. (2002) and counted in at least 20 different microscopic fields with a minimum of 1,000 cells in each condition.

**Immunocytochemistry and Immunohistochemistry**

For AMH and filipin III (Sigma) immunofluorescence, cultured cells were fixed for 10 min in 4% paraformaldehyde–PBS, washed with PBS, and incubated in 0.1 M glycine–PBS for 15 min. For cholesterol staining, cells were washed, incubated with filipin for 2 h at room temperature in the dark, washed again, and observed at fluorescence microscopy. For AMH staining, after glycine incubation, cells were washed and permeabilized for 20 min with 0.15% Triton X-100 (wt/vol; Sigma) in PBS containing 1% BSA. Nonspecific binding sites were blocked by incubating in 2% BSA–PBS for 20 min. Cells were then incubated for 60 min with antichicken AMH antibody (diluted at 1:200; Sekido and Lovell-Badge, 2007). The anti-AMH rabbit serum, produced by immunization with purified N-terminal His-tagged partial chicken AMH protein (from amino acid 66 to the C-terminus) was a gift of D. Carré-Eusèbe and E. Oréal (INSERM U782, Clamart, France). After another washing bath in PBS, AMH antibody was revealed with a goat anti-rabbit Alexa 488 antibody (1:500, 30 min; Invitrogen, Carlsbad, CA), and then cells were mounted with Vectashield (Vector Laboratories).

For testicular sections immunostained against AMH, testis from 4-wk-old chicken was fixed in formalin, paraffin-embedded, and sectioned (7 µm) as described in Froment et al. (2004). Sections were dewaxed and rehydrated in xylene in decreasing concentrations of alcohol (100, 90, and 75%). Antigen retrieval was performed by steaming the sections in a microwave in citrate buffer (0.01 M), pH 6.0, for 5 min, then cooling for 20 min after two 5-min washes in PBS. Sections were incubated at 4°C overnight with the rabbit anti-AMH antiserum diluted 1:200 in PBS containing 5% BSA (PBS–BSA). After washes in PBS–0.05% Tween 20 and PBS, slides were incubated in goat anti-rabbit Alexa 488 (diluted at 1:500 in PBS for 1 h). After three 5-min washes in PBS, sections were mounted with Vectashield. Negative controls were performed by replacing antibody with normal rabbit serum (data not shown).

**Reverse-Transcription PCR**

After 48 h of Sertoli cell culture, total RNA extraction and reverse transcription (RT) were performed as described (Froment et al., 2003). Amplification of chicken AMH (accession no. NM_205030.1) cDNA (310 bp fragment) used upper primer 5’ GTG GAT GTG GCT CCC TAC CC 3’ and lower primer 5’ CAG CAC CGA GGG CTC CTC C 3’ and amplification of chicken 3β-HSD (accession no. NM_205118.1) cDNA (400 bp fragment) used upper primer 5’ GCC AAG TTC CAG GGC AAC A 3’ and lower primer 5’ ACA GGT CAC AAG AAC CAC GCC T 3’. The PCR condi-
tions were 1 min denaturation at 94°C, 45 s annealing at 56°C, and 45 s extension at 72°C using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Control PCR with untranscribed RNA was performed in parallel (data not shown). The PCR amplification was stopped after 30 cycles before reaching the plateau. Water was used as negative control (first lane).

**Western Immunoblotting**

Cell lysates were prepared as described (Froment et al., 2003). Rabbit polyclonal antibody raised against human placental 3β-HSD (final dilution 1:500) was provided by V. Luu-The (Centre de Recherche en Endocrinologie Moléculaire, Quebec, Canada). Antibodies against phosphorylated extracellular signal-regulated kinase (ERK; Thr202/Tyr204) and phosphorylated Akt (Ser473) were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies were used at 1:1,000 dilution. Vinculin monoclonal antibody (hVIN-1, 1:1,000; Sigma) was used for normalization. As secondary antibodies, horseradish peroxidase-linked sheep anti-mouse IgG and donkey anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Antibodies were used at 1:1,000 dilution. Vinculin monoclonal antibody (hVIN-1, 1:1,000; Sigma) was used for normalization. As secondary antibodies, horseradish peroxidase-linked sheep anti-mouse IgG and donkey anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Antibodies were used at 1:1,000 dilution. Vinculin monoclonal antibody (hVIN-1, 1:1,000; Sigma) was used for normalization.

**Inhibin, Lactate, and cAMP Assay**

Inhibin concentration in the culture medium was measured using an enzyme immunoassay with inhibin α-subunit (1–32; human) EIA kit (Phoenix Pharmaceuticals Inc., Belmont, CA) according to the manufacturer’s recommendations and already tested in chickens (Hermannsson, 2007). All standards and samples were assayed in triplicate. Lactate concentration was determined using a commercial spectrophotometric assay purchased from Biomerieux (Marcy l’Etoile, France). Concentration of cAMP was measured by cAMP-Glo assay (Promega, Madison, WI) as recommended by the manufacturer. Values for each concentration are the mean ± SEM of at least 3 independent experiments.

**Statistical Analysis**

Data are presented as the mean ± SEM. Paired t-test was used to compare treated cells with the corresponding control. The effects on cAMP secretion, MTT, BrDU incorporation, and phagocytosis were analyzed using one-way ANOVA to test differences. If ANOVA revealed significant effects, the means were compared by Newman’s test, with \( P < 0.05 \) considered significant.

**RESULTS**

**Chicken Sertoli Cell Cultured In Vitro**

Chicken Sertoli cells present similarities with mammalian Sertoli cells (Figure 1A). The monolayers display a pavement-like morphology. Sertoli cells present distal ends of cytoplasmic processes as observed in mammalian Sertoli cells. Cells present high concentration of lipid droplets and granules, which are a characteristic feature of Sertoli cells in vivo. Lipid droplets are localized around the nucleus as observed after Filipin staining (Figure 1A). The nuclei of these cells exhibit the morphologic characteristics observed in Sertoli cells in vivo, with prominent nucleolus. The messenger of AMH, a well-known immature Sertoli cell marker in mammals and chicken (Tran et al., 1981), is present in chicken Sertoli cell culture (Figure 1B). The AMH protein is also detected by cytochemistry in Sertoli cells cultured in vitro (Figure 1C). As a positive control, AMH has been detected mainly in Sertoli cells in immature testis from 4-wk-old chicken.

After 24 h of culture, purity of the Sertoli cell preparation as determined by morphologic analysis and estimation of contaminant cells (germ cells and peritubular myoid cells) averaged 80 to 85%. Indeed, the percentage of peritubular myoid cell contamination, estimated by alkaline phosphatase staining, was close to 8% of the total cell population (8 ± 0.9%; n = 3; Figure 1D). Moreover, 3β-HSD enzyme expression and activity were detected in chicken Sertoli cells (Figure 1E, F). Similar results were observed in the positive control (chicken Leydig cells culture; Figure 1F). Stimulation of chicken Sertoli cells with eFSH (100 ng/mL for 48 h) upregulated expression of 3β-HSD (Figure 1G), suggesting that these cells are sensitive to gonadotropins in vitro.

**FSH Sensitivity of Chicken Sertoli Cells**

We investigated the sensitivity of the chicken Sertoli cells to FSH. Indeed, FSH stimulation in mammalian Sertoli cells activates 3 main downstream signaling pathways of the FSH receptor: the cAMP/protein kinase A, phosphatidylinositol 3-kinase/Akt, and the mitogen-activated protein kinase (ERK1/2) pathways (Crépieux et al., 2001; Khan et al., 2002; Froment et al., 2007).

Sertoli cells were stimulated at various times (0, 1, 5, 10, 30, and 60 min) with eFSH (100 ng/mL) treatment and with increasing eFSH concentrations (0, 1, 10, 20, 50, and 100 ng/mL) for 10 min (Figure 2A, B). As expected, FSH stimulation rapidly increased phosphorylation of Akt (tested on Ser473) and ERK (tested on Thr202/Tyr204) in chicken Sertoli cells (since 1 min) after FSH treatment (100 ng/mL; Figure 2A). Phosphorylations of Akt and ERK after 10 min of treatment were dose dependent, with an effect starting at 10 ng/mL (Figure 2B). In addition, a 10-min stimulation with
oFSH (100 ng/mL) or forskolin (10 µM) was able to induce an increase in cAMP concentration in the culture medium (Figure 2C).

**Proliferation of Chicken Sertoli Cells**

The ability of chicken Sertoli cells to proliferate in culture was investigated. During 7 d of culture, Sertoli cells were treated with IGF-1 (100 ng/mL), a well-known growth factor (Chandrashekar et al., 2004; Froment et al., 2007), or oFSH (100 ng/mL); in both cases, the number of viable Sertoli cells increased compared with untreated cells (Figure 3A). Under our culture conditions, chicken Sertoli cells were able to survive in the absence of FCS. As expected, 48 h of FSH treatment followed by a 24-h pulse of BrDU incorporation showed a 22% increase in cell proliferation (Figure 3B). As a positive control, incubation with IGF-1 increased the number of proliferating cells by about 87% compared with control (serum deprived cells). Interestingly, Sertoli cells prepared from prepubertal chickens (500 mg testis weight, obtained from 8.5-wk-old chickens) proliferated 2-fold more rapidly than Sertoli cells prepared from pubertal chickens (1,000 mg testis weight, obtained from 10-wk-old chickens; Figure 3C).

**Secretion and Phagocytosis Activity of Chicken Sertoli Cells**

Sertoli cells are known to produce several substrates such as lactate (an energy substrate for germ cells), hormones or growth factors (e.g., AMH, IGF-1, inhibin), and transport proteins such as androgen binding protein and transferrin. Lactate and inhibin secreted by chicken Sertoli cells were detectable in medium culture. After 48 h of FSH stimulation, the lactate concentration in culture medium increased about 1.6 fold (Figure 4A), and inhibin concentration tended to increase about 1.3 fold ($P = 0.1$; Figure 4B).

Phagocytosis of Sertoli cells eliminates the residual cytoplasm of germ cells during spermiogenesis (Carr et al., 1968). The phagocytic activity of chicken Sertoli cells has been evaluated as the ability to internalize latex fluorescent beads (1 µm) during the first 6 h of incubation. As shown in Figure 4C, chicken Sertoli cells engulfed 1 to 3 (50%) and 4 to 6 (37%) latex beads per cell. No phagocytic activity was detected in quail muscle cell line QM7, which was used as a negative control.

**Freezability of Chicken Sertoli Cells**

Primary chicken Sertoli cells were able to be frozen and thawed (Figure 5A). Moreover, chicken Sertoli cells were able to proliferate even after a maximum of 7 passages (Figure 5B). However, viability and proliferation of Sertoli cells were reduced. At passage 7 even after freezing–thawing, oFSH was still able to stimulate phosphorylation of ERK and Akt proteins (Figure 5C).
suggesting that chicken Sertoli cells retain their sensitivity to FSH. Finally, these cells could be cultured for up to 4 wk.

**DISCUSSION**

The aim of the present study was to establish a primary chicken Sertoli cell culture system and to investigate the functions of avian Sertoli cells in vitro. On the basis of morphological analysis, Sertoli cells of the domestic fowl present high similarities with Sertoli cells in mammals (Kodani and Kodani, 1966). Cells produced numerous projections and the cytoplasm contained numerous lipid droplets associated with high cholesterol levels. Sertoli cells are able to synthesize cholesterol (Maboundou et al., 1995). In addition, isolation of immature chicken Sertoli cells expressed AMH protein, known to be produced in immature Sertoli cells in others species (Tran et al., 1987). Even if most avian Sertoli cell features are similar to those of others mammals, purified chicken Sertoli cells expressed an active 3β-HSD in contrast to rodent Sertoli cells, as described previously (Woods and Domm, 1966; Cooksey and Rothwell, 1973). This enzyme is involved in steroidogenesis (Purohit et al., 1977; Nakabayashi et al., 1995) because it induces the conversion of cholesterol to pregnenolone or pregnenolone to progesterone.

A specificity of Sertoli cells is to produce lactate, an energy substrate for spermatocytes and spermatids, glycoproteins that facilitate transport such as transferrin, and androgen binding protein, and hormones such as IGF-1 and inhibin. In our experiments, chicken Sertoli cells secreted lactate and inhibin, which were upregulated in the presence of oFSH. These results are concordant with those described in mammal models (Mita et al., 1982; Toebosch et al., 1988). Another property of Sertoli cells is the phagocytic activity (Carr et al., 1968) that has been confirmed in our conditions.

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**Figure 3.** A) The number of viable chicken Sertoli cells during a 7-d period was determined indirectly by methylthiazolyldiphenyl-tetrazolium bromide assay. Sertoli cells were starved and then stimulated or not by ovine follicle-stimulating hormone (oFSH; 100 ng/mL) or insulin-like growth factor 1 (IGF-1; 100 ng/mL) for 7 d. Values are the mean ± SEM of 4 independent experiments performed in triplicate. B) Sertoli cells were labeled with bromodeoxyuridine (BrdU) for 24 h, after 48 h of oFSH (100 ng/mL) or IGF-1 (100 ng/mL) treatments. Cells were then stained with an anti-BrdU antibody and analyzed by indirect immunofluorescence. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (n = 5). Different letters indicate significant differences (P < 0.05). C) Sertoli cells prepared from 500 mg (germ cells in meiosis) or 1,000 mg (first spermatozoa are produced) testis were labeled with BrdU for 24 h (n = 4). Three asterisks (*** indicates P < 0.001.
by incubating cells with fluorescent beads and by presence of vacuoles observed in representative microscopic fields.

All these similar observations between cultures of mammalian Sertoli cells and chicken Sertoli cells demonstrated that the homogenous preparation of chicken cells comprised highly purified Sertoli cells. In addition, a short (10 min) stimulation induced phosphorylation of protein implied in the FSH pathway (ERK, Akt) and cAMP production, whereas a long FSH stimulation slightly increased proliferation, inhibin and lactate production, and 3β-HSD expression. These results strongly suggest that in vitro chicken Sertoli cells have conserved the sensitivity to FSH as rodent Sertoli cells (Steinberger et al., 1975). Interestingly, a similar response to oFSH (10–100 ng/mL) was observed in purified chicken Sertoli and mixed testicular cells as described by Rombauts et al. (1995).

To our knowledge, chicken Sertoli cells present more plasticity than mammal cells because they have a high proliferation rate in the presence of FCS and cells are able to be multiplied and passaged 7 times and can be frozen–thawed as a cell line. Thus, with 1 well of cell preparation, 64 wells of Sertoli cells could be obtained after 7 passages. These properties of chicken Sertoli cells are useful to analyze the effect of molecules with dose response and time course experiments.

In the rat model, Orth et al. (1988) demonstrated that the Sertoli cell number is critical to determine the testis weight and the sperm production because each Sertoli cell supports a relatively fixed number of germ cells for that species (Orth et al., 1988; Holsberger et al., 2005). Hence, deletion of 2 genes coding inhibitors of the cell cycle in Sertoli cells (the cyclin-dependent kinases inhibitors p27Kip1 and p21Cip1) increases proliferation, Sertoli cell populations, testis weight, and

Figure 4. A) Lactate and (B) inhibin concentrations in the culture medium were measured 48 h after ovine follicle-stimulating hormone (oFSH; 100 ng/mL) stimulation. Values (percentage of control) are the mean ± SEM of 4 independent experiments. Asterisk (*) indicates P < 0.05. C) Left panel: representative microscopic fields of the phagocytic activity of chicken Sertoli cells, and negative control using quail muscle cell line, QM7. Cells were in presence of latex fluorescent beads (1 µm) for 6 h, then were washed and fixed. The percentage of cells with number of internalized latex beads was counted and reported in different class (right panel). Different letters indicate significant differences. Asterisk (*) indicates P < 0.05; n = 3. Color version available in the online PDF.
the daily sperm production (Holsberger et al., 2005). In prepubertal chickens (4–8 wk of age), Sertoli cells are highly proliferative because the total number per testis increases to 1 to 5 million at 1 d of age to more than 100 million Sertoli cells at 8 to 10 wk of age (de Reviers et al., 1980; Sauveur, 1988). This in vitro Sertoli cell culture could be used to test nutritional factors that can affect Sertoli cell proliferation and consequently limit germ cell production and testis development.

In our experiment, we also showed that Sertoli cells prepared from prepubertal chickens (500 mg testis weight) were more proliferative than cells prepared from pubertal chickens (1,000 mg testis weight). These results supposed that in a more differentiated testis, Sertoli cells lose a part of their proliferative performance in favor of differentiation. Bozkurt et al. (2007) demonstrated by in vivo BrDU incorporation that the only cells still labeled after 8 wk were germ cells, indicating that Sertoli cell proliferation had ceased. Nevertheless, in vitro adult chicken Sertoli cells conserve some plasticity, and a preliminary analysis in our laboratory indicated that Sertoli cells prepared from an adult 40-wk-old chicken can proliferate in cell dishes (our unpublished data).

In conclusion, in the perspective of developing new, efficient methods (fast designed for the target, low cost, alternative to animal experiments), the culture system of chicken Sertoli cells presents several qualities that are complementary to existing mammalian approach. Indeed, avian Sertoli cells actively proliferate in vitro and can be passaged several times (7 passages before reaching senescence) and frozen in nitrogen. These physiological properties make chicken Sertoli cells highly suitable for easy and repeatable assays with fewer animals than can be conducted with mammalian Sertoli cells. A direct consequence of this methodological approach is to gain a deeper understanding of molecular mechanisms of chicken Sertoli cells activity and to determine whether xenобiotic molecules (i.e., pesticides) engendered alteration of Sertoli cells in vivo, leading to testicular dysfunction.

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