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Original article

Culture of porcine spermatogonia: effects of purification of the germ cells, extracellular matrix and fetal calf serum on their survival and multiplication

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Abstract — Initial studies to establish an in vitro system allowing survival and multiplication of porcine spermatogonia are described. Purified spermatogonia from 3-week-old pigs were cultured for 9 days alone or in the presence of Sertoli cells in either control medium or in medium supplemented with 5% fetal calf serum (FCS). Under either condition the number and the viability of the cells decreased with time, but both parameters were positively influenced by the presence of FCS. However, very few, if any, spermatogonia were able to take up BrdU under either condition. In another series of experiments, small fragments of seminiferous tubules from 3-week-old pigs were cultured in the presence of FCS, or seeded on an extracellular matrix. Under these conditions the number of cells decreased between day 0 and day 2 or day 5, then it remained roughly constant until the end of the culture. The number of spermatogonia decreased 2.5 fold during the two-week culture period. Spermatogonia were able to incorporate BrdU until the end of the experiment. The number of BrdU-labeled spermatogonia was higher when tubule-segments were seeded on an extracellular matrix. Then, the effects of the association of FCS and extracellular matrix were tested. The number of spermatogonia, during the whole culture period, was higher in serum-containing cultures than in serum-free cultures. As for the number of spermatogonia able to incorporate BrdU at different days, is decreased 3 fold between day 2 and 14 irrespective of the culture conditions. By contrast, the number of spermatogonia, labeled with BrdU between day 1 and 2, measured on days 5 to 14 of culture, was higher in serum-containing cultures. Finally, the number of spermatogonia labeled between day 1 and 2 was higher from day 5 onward than the number of spermatogonia able to take up BrdU between days 4 and 13. Taken together, these results indicate that intercellular communication and extracellular matrix are important for spermatogonia multiplication and that FCS promotes the survival of spermatogonia under in vitro conditions.

spermatogenesis / stem cells / in vitro

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Résumé — Culture des spermatogonies porcines : effets de la purification des cellules germinales, d'une matrice extracellulaire et du sérum de veau fœtal sur leur survie et leur multiplication. Cet article présente les premières études réalisées pour mettre en place un système de culture permettant la survie et la multiplication des spermatogonies de porc. Des spermatogonies purifiées, issues de porcelets âgés de 3 semaines ont été cultivées pendant 9 jours, soit seules, soit en présence de cellules de Sertoli, dans un milieu chimiquement défini, additionné ou non de 5 % de sérum de veau fœtal (SVF). Dans ces deux conditions, le nombre et la viabilité des cellules diminuent au cours de la culture, mais ces deux paramètres sont positivement influencés par la présence de SVF ; par contre, aucune spermatogonie n'est capable d'incorporer du BrdU. Dans une autre série d'expériences, de petits fragments de tubes séminifères de porcelets de 3 semaines ont été cultivés en présence de SVF ou sur une matrice extracellulaire. Le nombre de cellules décroît entre les jours 0 et 2 ou 5 puis reste constant jusqu'à la fin de l'expérience. Le nombre de spermatogonies diminue 2,5 fois au cours des deux semaines de culture quelles que soient les conditions de celle-ci. Par contre des spermatogonies sont, dans ces conditions, capables d'incorporer du BrdU à tous les jours de la culture ; le nombre de celles-ci est plus grand lorsque la culture est réalisée sur une matrice extracellulaire. Les effets de l'association d'une matrice et du SVF ont alors été recherchés. Tout au long de la culture, le nombre de spermatogonies est plus élevé en présence de sérum ; quant au nombre de spermatogonies capables d'incorporer du BrdU, il diminue de 3 fois entre les jours 2 et 14, que le milieu contienne du SVF ou non. Par contre, le nombre de spermatogonies ayant incorporé du BrdU entre les jours 1 et 2, mesuré aux jours 5 à 14 de la culture est plus élevé en présence qu'en absence de sérum. Enfin, le nombre de spermatogonies marquées entre les jours 1 et 2, mesuré à partir du jour 5, est plus élevé que celui des spermatogonies capables d'incorporer le BrdU aux jours 4 à 13 de la culture. Ces résultats indiquent que des contacts intercellulaires étroits et qu'une matrice extracellulaire sont des paramètres importants pour la multiplication des spermatogonies, et que le SVF permet une meilleure survie des spermatogonies en culture.

spermatogenèse / souches / in vitro

1. INTRODUCTION

Spermatogenesis starts with the proliferation of spermatogonia. This step takes place in the basal tubular compartment. The number of spermatogonial generations between stem spermatogonia and preleptotene spermatocytes determines the yield of spermatozoa; it varies among species. In the rat, the differentiating spermatogonia include six generations of proliferating cells. Hence, theoretically 1 024 primary spermatocytes can be formed from a single stem cell [25]. However, fewer are produced because of cell loss which has been calculated to result in a deficiency of between 25 and 75% in potential sperm production [7, 13, 14, 22]. In bovine, Kramer et al. [16] have described five peaks of spermatogonial mitoses yielding 8.3–17.8 primary spermatocytes arising from one stem cell. However, little is known about the regulation of proliferation and/or degeneration of spermatogonia in mammals [9]. Many growth factors: transforming growth factor β, inhibins, activins, follistatin, IGFs, and cytokines: IL1, IL6, TNFα, Stem cell factor (for a review see [20] and references therein) are produced by the Sertoli cells. However, for most of these factors, their function in spermatogenesis remains unknown. Therefore, an in vitro model system would be useful in order to investigate their potential role in mitosis, survival and differentiation of spermatogonia. Moreover, the culture, the multiplication and the transfection of spermatogonia, then the differentiation of these cells into spermatozoa, or even round spermatids, either in vivo [1, 3] or in vitro [15, 31] might lead to new approaches for the production of transgenic animals.

The use of pig organs for transplantation to human is becoming increasingly necessary.

However, pigs organs must be genetically modified in order to prevent (or delay) their rejection. For instance, it has been shown that the organs from pigs expressing human DAF or CD59 genes are rejected more slowly than those from control animals [6].

In the present report, we describe initial studies to set up an in vitro system allowing survival and multiplication of porcine spermatogonia; the results show the importance of fetal calf serum and extracellular matrix, respectively, in these processes.

2. MATERIALS AND METHODS

2.1. Isolation and purification of spermatogonia

Testes from 3-week-old Meishan pigs were used in these experiments. Pigs were anesthetized by electronarcosis then bled. Testes were quickly excised, decapsulated, minced into small pieces; then they were incubated in DMEM/F12 (1:1) medium pH 7.4 containing 0.75 mg·mL^{-1} collagenase (Serva; Biowhittaker, Fontenay-sous-Bois, France), 0.01 mg·mL^{-1} DNAse, 1 µg·mL^{-1} soybean trypsin inhibitor (both from Sigma, La Verpillière, France), 15 mM Hepes, 1.2 g·L^{-1} $NaHCO_3$ and antibiotics, for 1 h at 33 °C in a shaking water bath. After 3 washes in DMEM/F12 medium, seminiferous cord fragments were incubated in DMEM/F12 medium containing 0.5 mg·mL^{-1} trypsin (Sigma) for 5 min in the conditions described above. At the end of the digestion period, 0.1 mg·mL^{-1} soybean trypsin inhibitor and 0.2 mg·mL^{-1} DNAse were added. The dispersed cells were washed once with medium and filtered through a 20 µm nylon mesh (Alias Technologie, La Ravoire, France). In some experiments, aggregated Sertoli cells retained on the filter were collected and used for coculture of spermatogonia and Sertoli cells (see below). The dissociated cells were then separated on a discontinuous density gradient. An iso-osmotic solution containing 90% Percoll (Pharmacia Biotech, Les Ulis, France) in HAM/F10 medium (Life Technologies, Cergy-Pontoise, France) supplemented with 15 mM Hepes, $NaHCO_3$ (1.2 g·L^{-1}), BSA (6 mg·mL^{-1}) and DNAse (45 µg·mL^{-1}), pH 7.4 was prepared. A discontinuous density gradient was made by diluting the iso-osmotic Percoll solution with Hepes-buffered HAM/F10 supplemented with BSA (7 mg·mL^{-1}) and DNAse (50 µg·mL^{-1}). The gradient was formed by two fractions with 20 and 40% Percoll. The cell suspension was applied on the top of the gradient, and spun at 1 250 × *g* for 25 min at 4 °C. Cells found at the interface of the two fractions were collected, spun down by low-speed centrifugation and then resuspended in Hepes-buffered DMEM/F12 medium. The enriched spermatogonial fraction was then subjected to differential plating to eliminate contaminating somatic cells. The cells were incubated in DMEM/F12 medium containing 10% fetal calf serum for 12 h at 33 °C. Sertoli and myoid cells attached to the culture plates. The spermatogonia cells, which remained in suspension, were collected and washed in DMEM/F12 before seeding (day 0 of experiment). The purity of the cell preparation was determined using vimentin immunostaining (see below).

2.2. Culture of purified spermatogonia

Cell populations enriched in spermatogonia were placed in 2 cm^2 plastic wells at a density of 2×10^5 cells·cm^{-2}. Cells were then cultured in A medium: Hepes-buffered DMEM/F12 (1/1) medium (pH 7.4), supplemented with insulin (10 µg·mL^{-1}), transferrin (10 µg·mL^{-1}), vitamin C (10^{-4} M), vitamin E (10 µg·mL^{-1}), testosterone (10^{-7} M), retinoic acid (3.3×10^{-7} M), retinol (3.3×10^{-7} M), pyruvate (1mM) (all products from Sigma), and ovine NIH FSH-20 (1 ng·mL^{-1}) obtained through NIADDK (lot n° AFP-7028D) [15] in the absence or presence of 5% fetal calf serum. Incubation was

carried out at 33 °C in a water-saturated atmosphere of 95% air: 5% CO_2. The medium was changed every other day with great care, in order to not detach poorly adherent cells. At selected days of culture, spermatogonia were detached from culture dishes with a solution of 1 mM EDTA in PBS. An aliquot of the cell suspension was used to determine the number of cells and to assess cell viability by trypan blue exclusion (final concentration 0.02%, 5 min). Another aliquot was fixed with ice cold 70% ethanol for determination of the purity of spermatogonia by vimentin immunostaining (see below).

2.3. Coculture of spermatogonia and Sertoli cells

TR-M cells, a myoid cell line (a generous gift of Dr J.P. Mather [18]), were used for the preparation of an extracellular matrix. These cells were seeded in bicameral chambers (Falcon, Becton-Dickinson Europe, Meylan, France) at a density of 2×10^4 cells·cm^{-2} and cultured in Hepes-buffered DMEM/F12 supplemented with penicillin-streptomycin 10 000 IU·mL^{-1} (Gibco-BRL, Life Technologies), nystatin 10 000 IU·mL^{-1} (Sigma) and 5% fetal calf serum until confluence. Cells were then removed by treatment with 0.02 M NH_4OH for 5–15 min followed by3 to 5 washes with PBS. The extracellular matrix was then stored in PBS at 4 °C until used.

The fraction of aggregated Sertoli cells obtained at the time of spermatogonia preparation (see above) was washed. The cells were seeded on the extracellular matrix at a density of 3×10^5 cells·cm^{-2} in medium A (see above) supplemented with 0.2 % fetal calf serum; 12 h later, the purified spermatogonia were seeded (3×10^5 cells·cm^{-2}) on the Sertoli cell layer. After 2 days of culture, media in apical and basal compartments were replaced by A medium without fetal calf serum, then the medium in the basal compartment was changed every other day. At selected days of culture, cells were detached from culture dishes by trypsinization. An aliquot of the cell suspension was used to determine the number of cells and to assess cell viability by trypan blue exclusion. Another aliquot was fixed by ice-cold 70% ethanol for determination of the proportion of spermatogonia by vimentin immunostaining (see below).

2.4. Preparation and culture of tubule fragments

Testes of 3-week-old Meishan pigs were also used in these experiments. Testes were excised, decapsulated, and minced into small pieces (diameter about 100 μm) ; then they were suspended in Hepes-buffered DMEM/F12 medium (pH 7.4) containing 0.8 mg·mL^{-1} collagenase, 0.01 mg·mL^{-1} DNAse, 1 μg·mL^{-1} soybean trypsin inhibitor and antibiotics, for 1 h at 33 °C in a shaking water bath. The minced pieces of testis were allowed to sediment for 10–15 min, and tubule fragments were collected, then cut into small pieces with two lancets. These tubular fragments were washed and then gently resuspended in culture medium.

Cell samples were seeded at about 5×10^5 cells·cm^{-2} in bicameral chambers coated or not with an extracellular matrix (see above) in medium A without or with 5% fetal calf serum. After 2 days of culture, media in apical and basal compartments were replaced; then only the medium in the basal compartment was changed every other day. At selected days of culture, cells were detached from culture dishes by trypsinization (trypsin 0.05%, EDTA 0.02%, Gibco-BRL, Life Technologies) 10 min. An aliquot of the cell suspension was used to determine the number of cells and to assess cell viability by trypan blue exclusion. Another aliquot was fixed by ice-cold 70% ethanol for determination of the proportion of spermatogonia by vimentin immunostaining (see below).

2.5. Incorporation of BrdU

At selected days of culture, 1 μM 5-bromo-2'-deoxyuridine (BrdU, Sigma) was added to the apical medium. After 24 h of incubation, the apical and basal media were replaced. The BrdU-labeled cells were then revealed by immunostaining (see below).

2.6. Immunocytochemical studies

Cell suspensions fixed by ice-cold 70% ethanol for at least 24 h were cytospun on 3-aminopropyltriethoxysilane pretreated slides (5×10^4 cells per slide) and rehydrated in PBS. Two immunocytochemical reactions were successively performed.

1. To discriminate between somatic cells and germ cells, immunocytochemical reaction against vimentin (solely expressed by somatic cells) was performed as follows: the cells were permeabilized with Triton X-100 0.5% in PBS for 5 min. Then, the cells were incubated with 3% hydroxide peroxide for 5 min, in a humidified chamber at room temperature, rinsed with PBS, then incubated for 10 min with a mouse monoclonal antibody (clone v9), which recognizes vimentin filaments (at 1:1000 dilution in antibody diluent). After washing with PBS, the cells were incubated with a second biotinylated antibody (multilink-biotin anti Goat, Mouse and Rabbit antibody) for 10 min (at 1:150 dilution in antibody diluent). The cells were washed for 10 min in PBS. The staining reaction was performed with streptavidin-biotinylated horseradish peroxidase (StrapABC Complex/HRP) and diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) as chromogen (all products from Dako, Trappes, France).

2. A similar protocol was performed for revelation of BrdU, but denaturation of DNA was required. Cells were incubated for 5 min with 0.07 N NaOH diluted in alcohol:water (v:v); then they were dipped in PBS for 10 min. Cells were incubated with a monoclonal mouse anti-bromodeoxyuridine antiserum (Dako) at 1:500 dilution in a humid chamber at 4 °C overnight. After washes, the staining reaction was performed with streptavidin-biotinylated horseradish peroxidase and Vector-VIP (Valbiotech, Paris, France) as chromogen. Slides were dehydrated and mounted.

Cultured cells were fixed directly in bicameral chambers with Bouin's fixative for 20 min at room temperature. After 5 washes with PBS, the 2 immunocytochemical reactions were successively performed as above and nuclei were counterstained with Harris hematoxylin. The bottom of the insert was cut off with a scalpel, dehydrated and mounted.

2.7. Identification of germ cells

Testes of 3-week-old Meishan pigs were fixed in Bouin's fluid for 12–24 h and then embedded in paraffin. Five micrometer thin sections were deparaffinized and rehydrated, then incubated with a monoclonal mouse anti-vimentin antibody as above. The staining reaction was performed with AEC as chromogen and nuclei were counterstained with Mayer's hematoxylin. Spermatogonia were recognized by their absence of labeling by the anti-vimentin antibody and by their large spherical nucleus containing a finely granular and weakly stained chromatin.

At selected days of culture, identification of germ cells was performed directly in the bicameral chamber as above. At the end of cultures, some preleptotene spermatocytes could be occasionally identified by their nucleus which was smaller and more stained than that of spermatogonia. However, less than one cell of this type per 800 germ cells was observed. Hence, all the germ cells present in the cultures were considered as spermatogonia.

2.8. Quantification of total and BrdU-labeled spermatogonia

Immunostained cells were examined under light microscopy with an Axioscope

microscope (Carl Zeiss, Oberkochen, Germany). Two measures were performed: first, the percentage of spermatogonia in the cultures was determined (at least 500 cells were counted, in duplicate samples); second, the percentage of BrdU-labeled spermatogonia (vs. the total number of spermatogonia) was determined (at least 300 spermatogonia were counted, in duplicate samples). The number of total spermatogonia was obtained by multiplying the percentage of spermatogonia by the total number of cells (at least two measurements of the total number of cells were performed, in duplicate samples) at each day. The number of BrdU-labeled spermatogonia was obtained by multiplying the percentage of BrdU-labeled spermatogonia by the total number of spermatogonia.

2.9. Statistical analysis

Analysis of variance followed by the Bonferroni/Dunn a posteriori test or PLSD test of Fisher were used throughout.

3. RESULTS

In paraffin sections of testes from 3-week-old Meishan pigs, germ cells were identified by their negative reaction with the anti-vimentin antibody. Only spermatogonia were observed; they were most often localized near the basement membrane of the tubules (Fig 1). Therefore, in the first series of experiments, spermatogonia were purified from 3-week-old pigs (Fig. 2), then they were cultured for 9 days in either control medium or in medium supplemented with 5% fetal calf serum. At selected days of culture, the number and the viability of cells were determined together with the proportions of spermatogonia in the culture (Tab. I). Under both conditions, the number of cells decreased steadily with time. At the end of the experiment, the number of cells maintained in the presence of serum, which represented 40% of the number of cells seeded, was about two-fold higher than that of cells cultured in serum-free medium. Likewise, the viability of the cells was always higher in the presence of serum than in its absence ($p < 0.01$). The percentage of spermatogonia remained roughly constant throughout the experiment and was not influenced by the presence of serum in the culture medium. The small increase of this percentage between day 0 and day 2 most probably was due to platting of some contaminating somatic cells which were not detached from the dishes by PBS-EDTA (see Materials and Methods section).

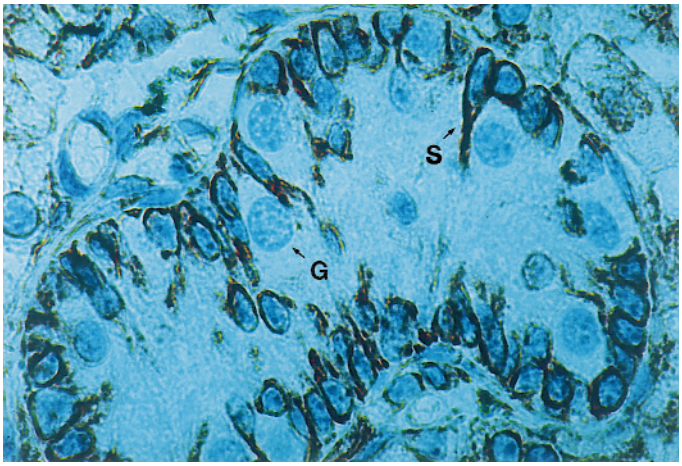

Figure 1. Photomicrograph of a section from the testis of a 3-week-old Meishan pig. The section was incubated with an anti-vimentin antibody and counterstained with Mayer's hematoxylin. S: Sertoli cell. G: spermatogonia. Magnification: × 1 000*.

* This figure is available in colour at www.edpsciences.org

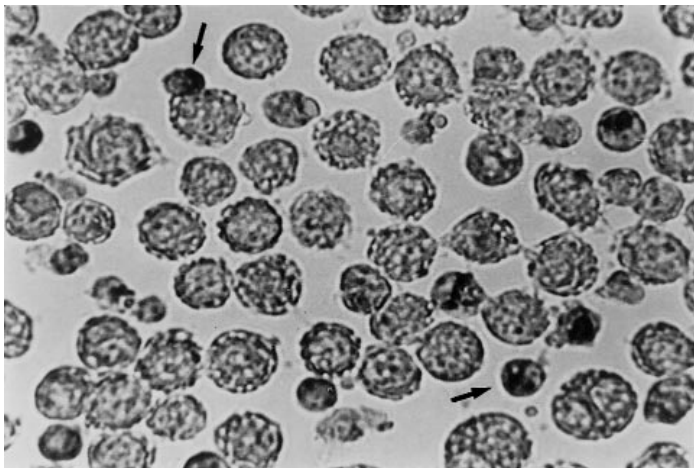

Figure 2. Photomicrograph of cytospun freshly purified porcine spermatogonia. The cells were incubated with an anti-vimentin antibody and counterstained with Mayer's hematoxylin. Somatic cells (arrow) are labeled by the anti-vimentin antibody. Magnification: × 1 250*.

Table I. Changes in the cell number and viability and in the percentage of spermatogonia during culture of purified preparations of porcine spermatogonia in the absence or presence of 5% fetal calf serum (FCS).

Days of culture	Serum-free medium A			Medium A supplemented with 5 % FCS		
	Number of cells	Viability (% of total cells)	Spermatogonia (% of total cells)	Number of cells	Viability (% of total cells)	Spermatogonia (% of total cells)
Day 0	400 000	91	78	400 000	91	78
Day 2	174 000	78	88	243 000	86	93
Day 5	159 000	77	94	190 500	88	95
Day 7	147 000	76	89	175 500	87	90
Day 9	77 250	78	92	159 000	89	93

Results are the mean of duplicate determinations. Similar results were obtained in another experiment.

In order to determine whether spermatogonia cultured under those conditions could enter the S phase of the cell cycle, BrdU was added to the medium on day 1, and the number of BrdU-labeled germinal cells was counted on day 2. Very few, if any (less than 1%), spermatogonia were labeled under either culture condition (data not shown).

Hence, in the second series of experiments we looked for an effect of Sertoli cells on the ability of spermatogonia to incorporate BrdU. Therefore, purified spermatogonia were cultured on Sertoli cells isolated from the same animals. In order to help Sertoli cells to plate, they were seeded on an extracellular matrix prepared as described in the "Materials and Methods" section and cultured either in the absence or presence of 5% fetal calf serum. Under serum-free conditions, the number of total cells decreased during culture in such a way that on day 9 only 37% of seeded cells were still present (Tab. II). Likewise, the viability of the coculture and the percentage of spermatogonia decreased with time, thereby indicating that both spermatogonia and

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Table II. Changes in the cell number and viability and in the percentage of spermatogonia during coculture of purified porcine spermatogonia with Sertoli cells in the absence or presence of 5% fetal calf serum (FCS).

Days of culture	Serum-free medium			Medium supplemented with 5 % FCS		
	Number of cells	Viability (% of total cells)	Spermatogonia (% of total cells)	Number of cells	Viability (% of total cells)	Spermatogonia (% of total cells)
Day 0	300 000	91	39	300 000	91	39
Day 2	146 400	82	59	127 300	86	58
Day 5	128 400	72	36	117 600	71	33
Day 7	108 000	63	22	174 600	63	16
Day 9	111 600	55	24	174 000	60	16

Results are the mean of duplicate determinations. Similar results were obtained in another experiment.

Sertoli cells were lost during the experiment. Addition of fetal calf serum to the culture medium resulted in a number of total cells, at the end of the culture, which was higher than under serum-free conditions. However, the number of spermatogonia was similar, during the whole culture period, under both conditions. Again, no BrdU-labeled germinal cells could be observed when BrdU was added to the culture medium between day 1 and day 2 (data not shown).

Therefore, in a third series of experiments, we chose to seed small fragments of seminiferous tubules, in order to determine whether tight intercellular contacts are important for spermatogonia survival and/or multiplication.

Tubule segments from 3-week-old pigs were either cultured in medium containing 5% fetal calf serum or seeded on an extracellular matrix and cultured in serum-free medium for 2 weeks. After seeding, cells migrated away from the tubule segments and spread out on the insert surface so that from day 2 or 3 of culture the shape of the tubules was no longer observed (Figs. 3a and 3b). The germinal cells were then placed on the surface of the layer formed by the Sertoli cells (Fig. 3c). The number of cells decreased quickly between day 0 and day 2 in serum-containing medium or between day 0 and day 5 in the absence of serum; then it remained roughly constant when cells were maintained in serum-free medium, or even increased slightly up to day 14 ($p < 0.05$) when cultures were performed in the presence of serum (Figs. 4a and 4b). Cell viability was similar under both conditions, and ranged between 62 and 87% of total cells counted. Likewise, the number of spermatogonia was never different irrespective of the conditions of culture; it decreased roughly 2.5 fold during the 2-week-culture period ($p < 0.05$) (Fig. 4c).

Spermatogonia cultured under these conditions were able to incorporate BrdU (added to the culture medium 24 h before stopping the culture on days 2, 5, 7, 11 and 14) until the end of the experiments (Figs. 3c and 5); however, the number of BrdU-labeled spermatogonia decreased throughout the culture period under both conditions ($p < 0.01$). Nevertheless, the number of BrdU-labeled spermatogonia was significantly higher when tubule segments were seeded on an extracellular matrix ($p < 0.01$); this resulted from differences on day 2 and day 5 of culture (both $p < 0.05$).

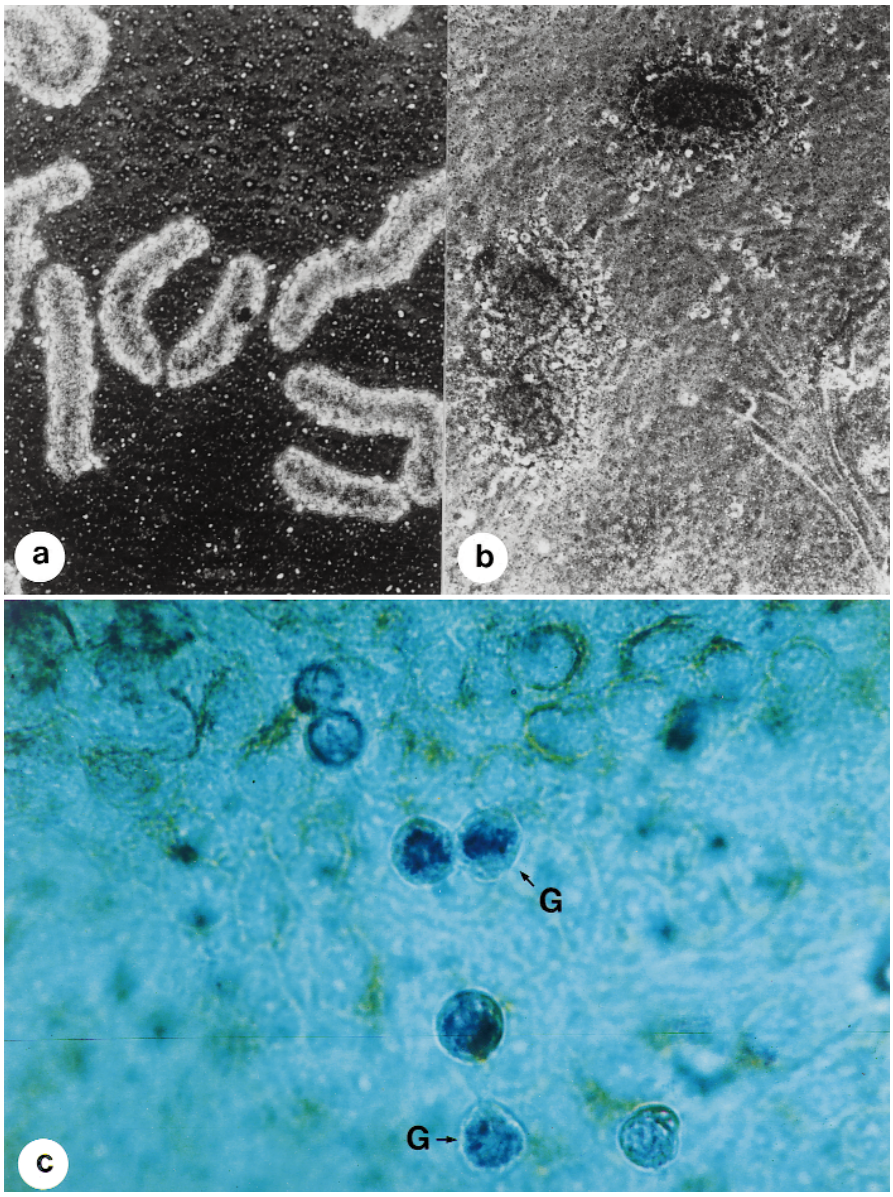

Figure 3. Microscopic aspect of seminiferous tubular segments (**a, b**) and testicular cells (**c**) from 3-week-old Meishan pigs in culture. (**a**) After seeding (day 0); (**b**) After 5 days of culture; (**c**) Cytochemical and immunocytochemical analysis of germinal cells on day 11 of culture. The cells were incubated with both anti-vimentin antibody (brown colour) and anti-BrdU antibody (violin colour) and counterstained with Harris hematoxylin. G: spermatogonia. Magnification: a and b × 100; c × 1 000*.

* This figure is available in colour at www.edpsciences.org

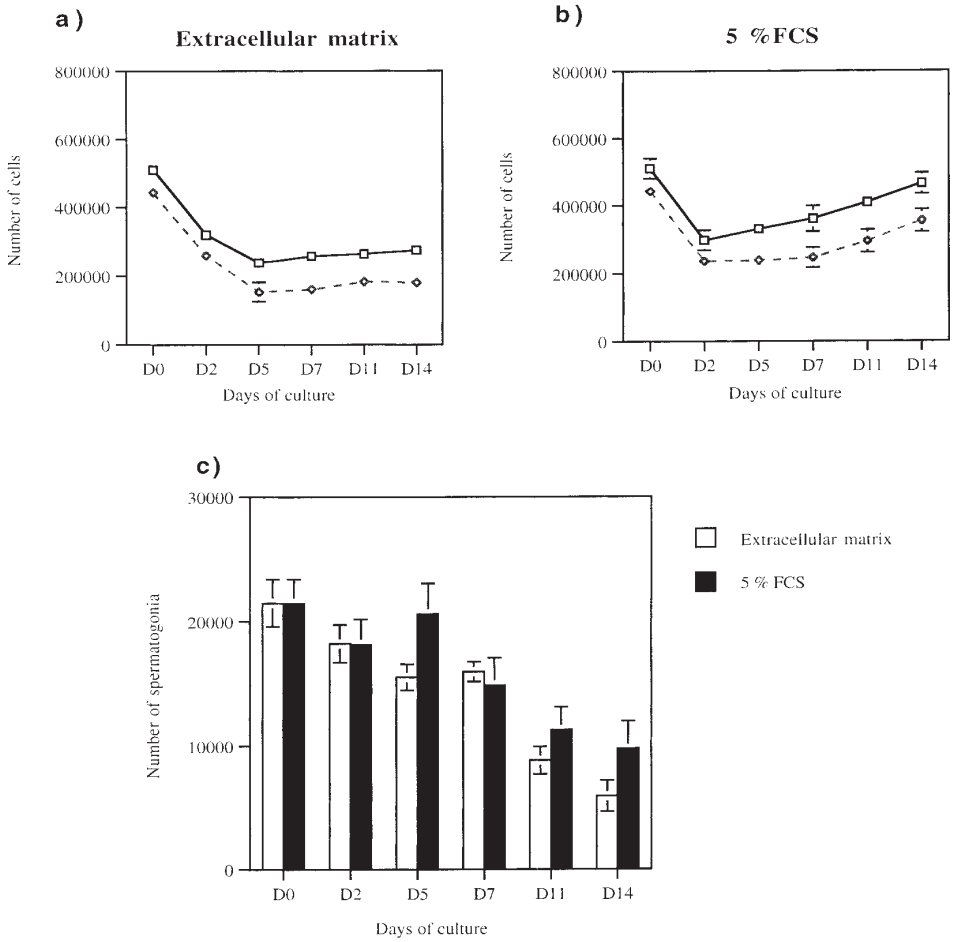

Figure 4. Changes in the number of total and viable cells in cultures of seminiferous tubular segments from 3-week-old Meishan pigs. (**a**) Total (□— —□) and viable (◆— —◆) cells were cultured in serum-free medium on an extracellular matrix. (**b**) Cells were cultured without cellular matrix in medium containing 5% fetal calf serum (FCS). (**c**) Number of spermatogonia in cultures performed on extracellular matrix (□) or in the presence of 5% fetal calf serum (FCS) (■). Results are the mean ± SEM of duplicate determinations in 4 different experiments.

Then, we tested the effects of the association of fetal calf serum and extracellular matrix. On day 14 of the experiments, the number of total cells was 66% and 82%, respectively, of that on day 0, for cells cultured in the absence or presence of fetal calf serum; cell viability decreased from 87 ± 2% on day 0 to 64 ± 5% and 68 ± 7% respectively on day 14. The number of spermatogonia, during the whole culture period, was higher in serum-containing cultures than in serum-free cultures ($p < 0.02$); it decreased 2-fold ($p < 0.05$) between day 0 and day 14 in cultures maintained in serum-free medium, but did not decrease significantly ($p > 0.05$), throughout the experiment, when serum was present (Fig. 6). Therefore, the uptake of BrdU by spermatogonia was investigated in two ways: (i) BrdU was added to the culture medium

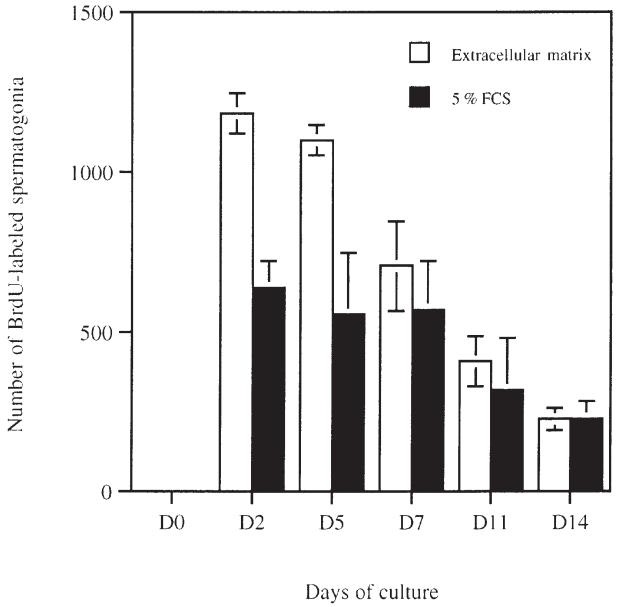

Figure 5. Changes in the number of spermatogonia able to take up BrdU in cultures of seminiferous tubular segments from 3-week-old Meishan pigs. BrdU (1 μM) was added to culture media 24 h before stopping the culture and the number of BrdU-labeled spermatogonia was determined as described in "Materials and Methods". Cells were cultured on an extracellular matrix in serum-free medium (□) or without cellular matrix in the presence of 5% fetal calf serum (FCS) (■). Results are the mean ± SEM of duplicate determinations in 3 different experiments.

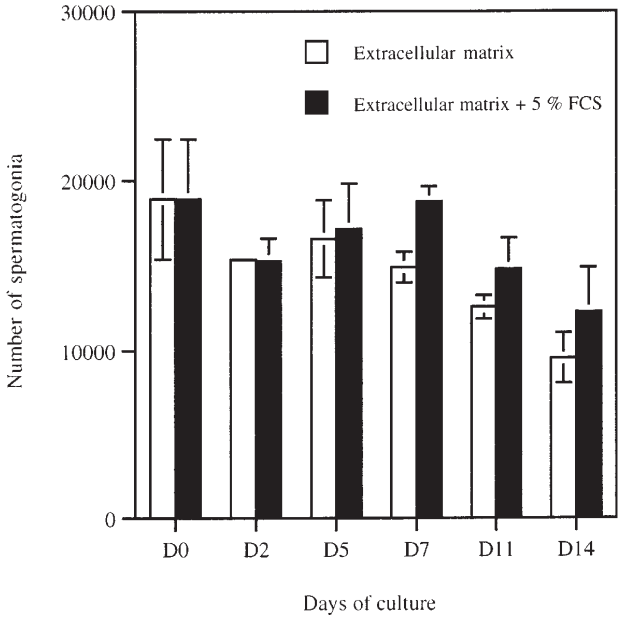

Figure 6. Changes in the number of spermatogonia in cultures of seminiferous tubular segments from 3-week-old Meishan pigs. Cells were cultured on an extracellular matrix in the absence (□) or presence (■) of 5% fetal calf serum (FCS). Results are the mean ± SEM of duplicate determinations in 3 different experiments.

24 h before stopping the cultures, as above; (ii) the number of spermatogonia having incorporated BrdU between day 1 and day 2 was determined on days 2, 5, 7, 11 and 14 of the experiment (Figs. 7a and 7b). The number of spermatogonia able to incorporate BrdU at different days of culture decreased 3-fold ($p < 0.05$) between day 2 and day 14 irrespective of the culture conditions. By contrast, the number of spermatogonia,

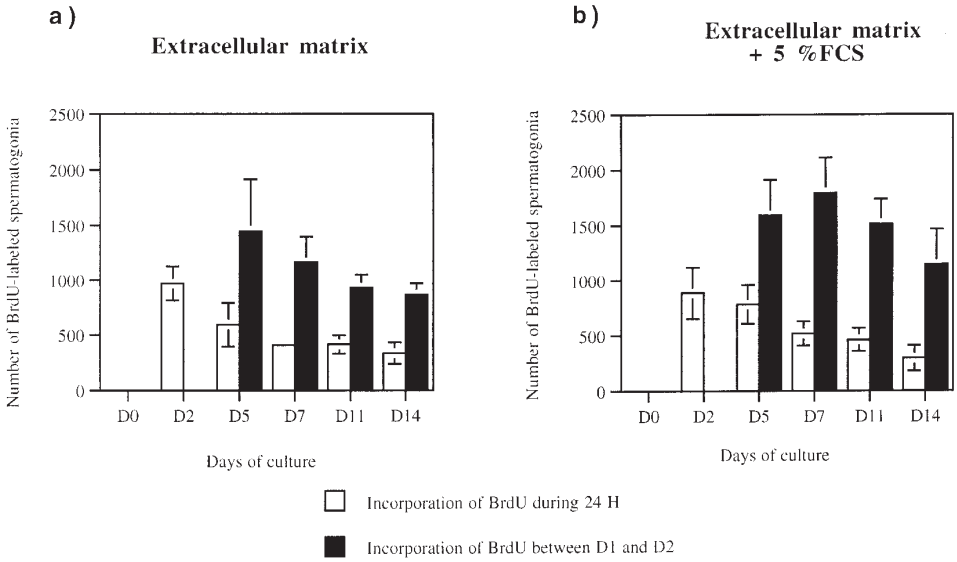

Figure 7. Changes in the number of BrdU-labeled spermatogonia in cultures of seminiferous tubular segments from 3-week-old Meishan pigs. BrdU (1 μM) was added to culture media 24 h before stopping the cultures (□) or added to culture media between day 1 and day 2 and the cultures stopped at selected days of the experiment (■). Cells were cultured on an extracellular matrix in the absence (**a**) or presence (**b**) of 5% fetal calf serum (FCS). Results are the mean ± SEM of duplicate determinations in 3 different experiments.

labeled with BrdU between day 1 and day 2, measured on days 5 to 14 of culture, was significantly higher ($p < 0.05$) in cultures maintained in the presence of serum than in its absence. Moreover, there was a significant increase in the number of those spermatogonia between day 2 and day 5 (from 886 ± 234 to 1594 ± 318, $p < 0.02$) in the presence of serum. Finally, it should be underlined that the number of spermatogonia labeled between day 1 and day 2 was higher from day 5 onward than the number of spermatogonia able to take up BrdU between days 4 and 13 ($p < 0.05$).

4. DISCUSSION

Many attempts to investigate the regulation of multiplication and differentiation of spermatogonia have been made, either in vivo [4, 5, 10, 11, 23] or in vitro [2, 19, 21, 27, 29, 30]. However, the factors which are involved in the regulation of these processes remain largely unknown. Moreover, all the in vitro studies published so far were performed in the rat or mouse species with only one report (to our knowledge) dealing with the survival of isolated porcine spermatogonia over a short period of culture [8]. The aim of the present studies was to compare different culture systems in order to select one system allowing multiplication of spermatogonia in vitro.

Purified spermatogonia, cultured either alone or even seeded on a Sertoli cell layer died rather quickly, since about 80% of these germ cells were lost over a 9-day culture period. This decrease in cell number was correlated with a decrease in cell viability, as assessed by trypan blue exclusion. This decrease in cell viability was more marked in germ cell/somatic cell cocultures than in spermatogonia cultured alone. At least two

reasons might explain this difference: (i) trypsinization of the cocultures was needed to remove cells from culture dishes in order to perform counting, whereas trypsin was not required for spermatogonia cultured alone; (ii) some dead spermatogonia remained attached to clusters of Sertoli cells and were therefore counted, whereas dead spermatogonia cultured alone were eliminated when removing the culture medium before adding PBS-EDTA (see Materials and Methods section) for detaching the viable cells. The decrease in viability, with time in culture, of spermatogonia cultured alone was less marked in the present work than in that of Dirami et al. [8]. This can be explained, only partly, by the different methods used to assess this parameter (MTT colorimetric assay in Dirami's work and trypan blue exclusion in our study) since the kinetics of this decrease was also slower in our work. Such a difference should be best explained by the culture media used in these experiments. Indeed, the medium used in our work was supplemented with vitamins, hormones and metabolic substrates; this was not the case in the study of Dirami et al. [8]. Besides, these authors have shown a positive effect of stem cell factor and GM-CSF on porcine spermatogonia survival. In addition, van Pelt et al. [30] have reported that spermatogonia from vitamin A deficient rat testis can be maintained in culture, for 3 days, with a viability of more than 95%, if serum from such rats is added to the incubation medium. This fits well with the better viability and survival of isolated germ cells cultured alone obtained in our study when fetal calf serum was included in the culture medium. However, no such effect of fetal calf serum was observed when spermatogonia were cultured on a Sertoli cell layer for 9 days. This fits rather well with the absence of effect of fetal calf serum during the first week of culture of seminiferous tubule segments (see below). Nevertheless, no incorporation of BrdU by purified porcine spermatogonia was ever observed in our experiments. This suggests that tight interactions of germ cells with somatic cells are important, in order to allow spermatogonia to enter the S phase of the cell cycle. In an attempt to keep these cellular interactions functional in vitro, in another series of experiments, small fragments of porcine seminiferous tubules were seeded. Under these conditions, some spermatogonia were able to take up BrdU, even after two weeks of culture, despite an overall decrease in the total number of spermatogonia present in the cultures. The percentage of spermatogonia able to incorporate BrdU in vitro was small (4 to 8%), but it was quite similar to the proportion of BrdU-labeled germ cells observed in the testis of 3-week-old pigs injected with BrdU one day before slaughtering (C. Marret, A. Locatelli and Ph. Durand, unpublished results).

Coating of the culture chambers with an extracellular matrix resulted in a higher number of spermatogonia taking up BrdU, during the first 5 days of experiment, than when fetal calf serum was added to the culture medium. However, the total number of spermatogonia was similar under both conditions. These results suggest that, at the beginning of the culture, the "exogenous" extracellular matrix promoted the entry into S phase of a subpopulation of spermatogonia, and that after several days, the somatic cells seeded had secreted a significant amount of matrix [26] making the differences between the two conditions of culture no longer significant. In support of such a hypothesis, it has been shown that in the absence of a matrix substrate, division of gonocytes from newborn rats is impaired in vitro [24].

Culture of seminiferous tubule segments on an extracellular matrix resulted in the maintenance of the number of spermatogonia, throughout the whole experimental period, only when fetal calf serum was added to the culture medium. This indicates that extracellular matrix and fetal calf serum have additive and/or complementary effects since, as opposed to the effects of extracellular

matrix (see above), those of fetal calf serum were observed only from the end of the first week of the experiment.

Fetal calf serum did not enhance the number of spermatogonia able to take up BrdU at any day of the experiment. However, the number of those spermatogonia having incorporated BrdU between day 1 and day 2 remained higher in serum-containing cultures than in serum-free cultures. Taken together these results reinforce the view (see above) that fetal calf serum promotes the survival of spermatogonia.

An interesting result which deserves further investigations is the multiplication, between day 2 and day 5, of spermatogonia labeled with BrdU between day 1 and day 2, and the lower number of spermatogonia able to take up BrdU between day 4 and day 5. This suggests that the germ cell population studied here is not homogeneous. The testis of pig contains several types of spermatogonia [28] as in other mammalian species such as the rat [12] or the Chinese hamster [17]. In these latter, the duration of the phases S and G2 of the cell cycle varies according to the different types of spermatogonia. In addition, the behaviour of spermatogonia under culture conditions may differ somewhat from that in vivo.

Another point which is not clear is why no meiotic cells appeared during the 2-week culture period. By contrast, Boitani et al. [2] in organ cultures of testes from 9-day-old rats, observed leptotene and zygotene spermatocytes after 2-weeks, when FSH was added to the culture medium. Therefore, additional studies are required to determine whether the maturity of the animals used in the present study or other parameters are responsible for these differences.

Nevertheless, these experiments have allowed us to define some parameters which are important for both the survival and the ability of porcine spermatogonia to proliferate under in vitro conditions. Thus, this culture system even with its imperfections may represent a useful tool to investigate further the regulation of these processes.

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