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Mitogenic effect of prepubertal rat Sertoli cell secreted media on germ and somatic cells in vitro

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In recent years it has been established that Sertoli cells secrete a number of bioactive molecules which participate in the autocrine and paracrine regulation of the testicular function. Cultured Sertoli cells were isolated from postnatal rat aged 6 and 12 days. The Sertoli cell-conditioned media (SCCM-6 and SCCM-12) can markedly stimulate the proliferation of BALB/c 3T3 somatic cells and quiescent rat prespermatogonia.

Key words: Sertoli cells, cell culture, mitogenic factor (s), secreted proteins, germ cell proliferation.

It is known that Sertoli cells are involved in paracrine interactions with germinal cells in mammalian testis, and secrete a number of growth factors required for control of germ cell function (B e l l v e, Z h e n g, 1989; L a m b, 1993). Some of these growth factors as Seminiferous growth factors (SGF), Sertoli cell-secreted growth factor (SCSGF), Transforming growth factor (TGF-α and TGF-β), acidic and basic Fibroblast growth factors (aFGF, bFGF), have been isolated and characterized. Cell-cell interactions in mammalian testis are dependent on the requirements of different cell types at each stage of testucular development (M a r t i n o v a et al., 1993). Germ cells enter a resting period, which lasts from 17th day p. c. until 5th day p. p. when the mitosis reinitiates (H i l s h e r et al., 1974), and on 12th day p. p. germ cells enter meiosis.

Cell-cell interactions as well as Sertoli cell structure and functions during early prepubertal period are not well studied. The aim of present work was to investigate the effect of proteins secreted by Sertoli cell derived from 6th-and 12th-day-old rats on germ and somatic cell proliferation in vitro.

Materials and methods

Dulbecco's modified Eagle medium (DMEM), Ham's F-12, fetal bovine serum (FBS), tissue culture dishes and 96-well multidishes were supplied by Flow labs., U.K.; trypsin from bovine pancreas 3,1 U/mg and dialysis tubing of 8000 MW exclusion limit were

supplied by Serva; collagenase was obtained from Boehringer, Mannheim, F. R. G.; hyaluronidase from Fluka; [^{35}S]-methionine and [^{3}H]-thymidine were purchased from Amersham.

Sertoli cells were isolated from 6- and 12-day-old male Wistar rats according to a modification of the procedures of B e l l v e (1979). Decapsulated testis fragments were digested first with hyaluronidase (0,5 mg/ml), followed by a collagenase digestion (0,5 mg/ml). Both digestions were performed for 15 min each at 32°C in a shaking water bath and additionally pipetted for 5 min. The dispersed seminiferous cords were isolated by allowing them to sediment for 10 min and supernatant was discarded. The sediment was digested with trypsin (0,5 mg/ml) for 10 min under the same conditions and then was pipetted for 3 min. The dispersed cell were washed with 0,5 % BSA and filtered through a wire mesh grid (74 μm pore size). Sertoli cells were plated in tissue culture dishes at a density of (1÷1,5).1 000 000 cells/ml in DMEM/ Ham's F-12 (1:1) supplemented with 5 % FBS during the initial 24 h of cultivation. The cell cultures consisted of 85±5 % viable Sertoli cells. After 24 h of cultivation the cells were washed with DMEM and fresh medium without serum was added. The Sertoli cell-conditioned medium (SCCM) was collected on the 4th and 7th day after beginning of the cultivation. Purity of Sertoli cells reached about 90 % after 4 days of culture, while the germ cells were completely absent.

Samples of SCCM from 6- and 12-day-old rats (SCCM-6 and SCCM-12) in concentrations 8 ×, 4×, 2 ×, and 1× were tested for their ability to stimulate DNA synthesis in confluent quiescent culture of BALB/c 3T3 cells. The fibroblasts were grown to confluence in 96-well polystyrene multidishes (2.1000 cells per well) in 200 μl of DMEM containing 10 % FBS for 3 days. Subsequently, the fibroblasts were cultured 7 days in DMEM containing 0,5 % bovine serum. Different samples of SCCM without serum were tested for a further 24h in the presence of [^{3}H]-thymidine (37 kBq per well).

Two-dimensional gel electrophoresis was performed essentially as described by O'F a r r e l l (1975) with some modifications. The putative factor(s) was pre-characterized as a protein with a molecular weight over 8000, and sensitive to heat and trypsin treatment (M a r t i n o v a et al., 1988).

In each experimental group were used five male rats on 2,5 and 3,5 days p.p. The animals were decapitated and the testes were dissected free of tunica albuginea to obtain four pieces from each testis. The pieces were mounted on a highly permeable celloidin membrane having a plastic ring as a frame (J o r d a n o v, A n g e l o v a, 1984). The incubation medium consisted of DMEM (in controls) and different concentrations of SCCM-6 and SCCM-12, supplemented with 5 % FBS and [^{3}H]-thymidine in a dose of 74 kBq/ml. The specimens were incubated at 32 °C in a humidified atmosphere of 95 % air and 5 % CO_2 for 24 h.

To assess the beginning of DNA synthesis by [^{3}H]-thymidine incorporation in the quiescent prespermatogonia, the germ cells were scored for the presence of labels. The labelled germ cells were counted in every 3rd section of each explant. The final result for each group (n=5) was determined by the mean value of triplicate incubations.

The percentage of seminiferous cords containing labelled germ cells was evaluated in the same manner.

Results

During a week of cultivation Sertoli cells preserved their morphological characteristics, growth in monolayer and form distinct groups of cells. The nucleus is large with

irregular shape and invaginations. The heterochromatin accumulations are situated on the inner nuclear membrane, and several dense nucleoli are visible. The chromatin structure and nucleoli demonstrated functional activity of the cells.

Electrophoretic examinations of the proteins secreted in the spent media from Sertoli cells are shown on Fig. 1. 20—25 different proteins with m. w. between 10 and 94 kDa and 5 and 9 pI are synthesized. There is no differences between secretory activity of Sertoli cells isolated from 6- and 12-day-old rats.

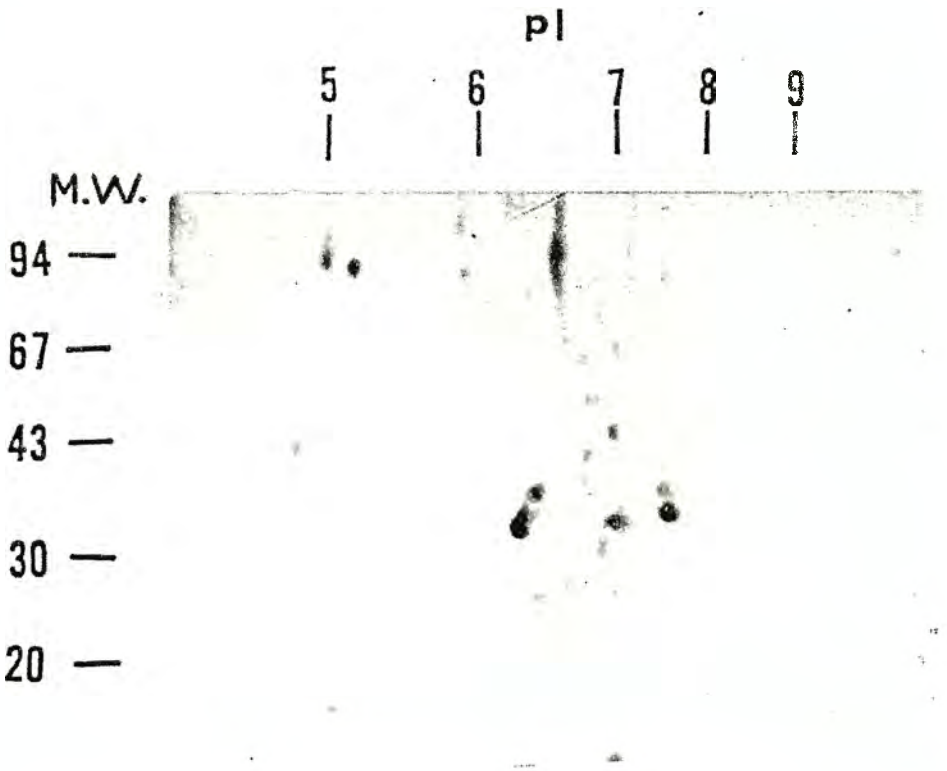

Fig. 1. Two-dimensional analysis of Sertoli cell secreted proteins.Cells were incubated in DMEM with [^{35}S]-methionine for 24h

The effect of different concentrations of SCCM-6 and SCCM-12 on the mitogenic activity of the BALB/c 3T3 cell line is shown in Fig. 2. SCCM-6 did not significantly increase cell proliferation of 3T3 cells in all tested concentrations (1×, 2×, 4×, respectively 10, 20, 40 μg/ml protein). A dose-response effect was observed in testing the same concentrations of SCCM-12. Maximum mitogenic effect was noted in 2× SCCM-12, when the [^{3}H]-thymidine incorporation was 7-fold over controls ($p<0,01$). The stimulation in the other concentrations was considerably high as well ($p<0,001$). In the group with heat treatment of 4 ×SCCM-12 a nearly 2-fold increase was registered in comparison with the control, but the differences were not significant ($p<0,25$). The mitogenic activity of SCCM-6 and SCCM-12 was trypsin sensitive (data not shown).

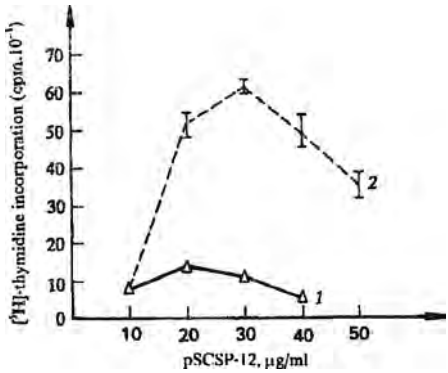

Fig. 2. Comparison between the effect of SCCM-6 (1×, 2×, 4×) and SCCM-12 (1×, 2×, 4×, 8×) on [³H]-thymidine incorporation by 3T3' fibroblasts in vitro
1 – 6 day; *2* – 12 day

Using the methods of seminiferous cord cultivation on the celloidin membrane we observed normal viability of prespermatogonia. From Fig. 3 it is evident that [³H]-thymidine is incorporated into the Sertoli cells as well as into prespermatogonia after incubation in SCCM-6 of testicular cords from a 3,5-day-old rat.

The effect of SCCM-6 and SCCM-12 on the mitotic activity of the germ cells from 2,5-day-old rats is expressed as percentage of seminiferous cords, containing labelled germ cells (Fig. 3) and as percentage of labelled germ cells (Fig. 4). SCCM-12 has no effect on the germ cells but SCCM-6 in a dose-dependent manner increases the investigated parameters up to 3-4-fold, compared to controls ($p<0,001$).

The mitogenic effect of SCCM-6 and SCCM-12 in the same concentrations is more expressed on the germ cells from 3,5-day-old rats (Fig. 5). Tested concentrations of SCCM-6 icrease significantly ($p<0,001$) up to 5-10-fold the percentage of seminiferous cords, containing labelled germ cells (Fig. 5) and the percentage of labelled germ cells (Fig. 6).

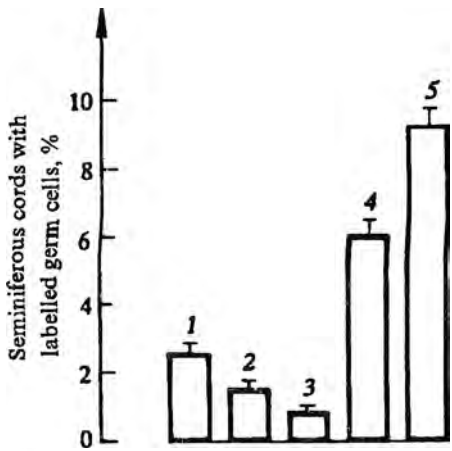

Fig. 3. Mitogenic effect of SCCM-6 and SCCM-12 on 2,5-day-old rat prespermatogonial cells. Seminiferous cords with labelled prespermatogonial cells
1 – DMEM (control); *2* – 4× SCCM-12; *3* – 2× SCCM-12; *4* – *4*× SCCM-6; *5* – 2×SCCM-6

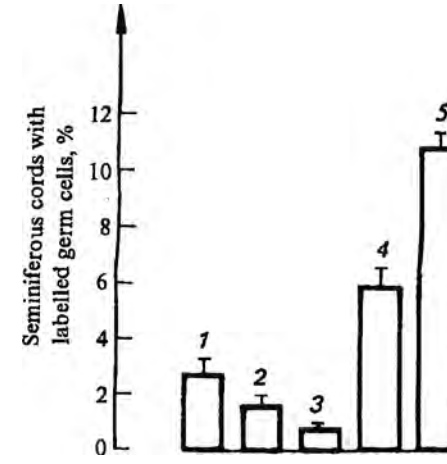

Fig. 4. Mitogenic effect of SCCM-6 and SCCM-12 on 2,5-day-old rat prespermatogonial cells. Percentage of labelled prespermatogonial cells
Designations as in Fig. 3

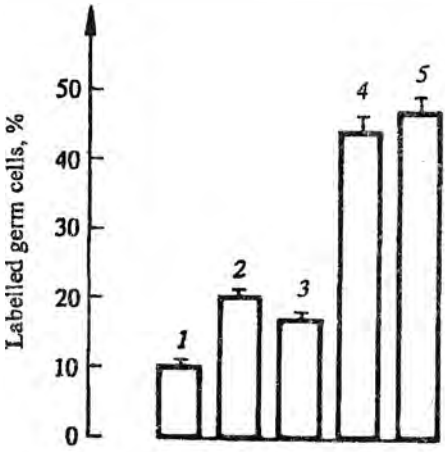

Fig. 5. Mitogenic effect of SCMM-6 and SCCM-12 on 3,5-day-old rat prespermatogonial cells. Seminiferous cords with labelled prespermatogonial cells
Designations as in Fig. 3

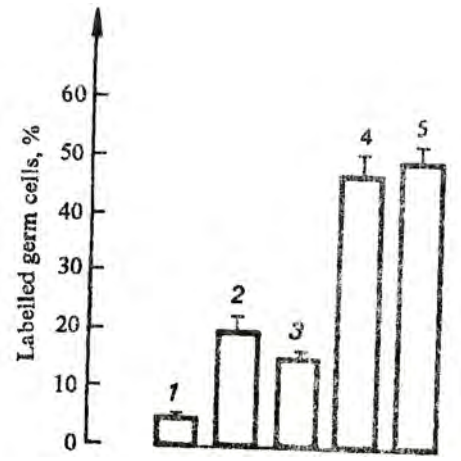

Fig. 6. Mitogenic effect of SCCM-6 and SCCM-12 on 2,5-day-old rat prespermatogonial cells. Percentage of labelled prespermatogonial cells
Designations as in Fig. 3

Discussion

In current study we have shown that Sertoli cell isolated from 6th- and 12th-day-old rat testis preserved their morphological and functional characteristics in vitro and secrete a number of products. Based on [^{35}S]-methionin labelling it is obvious that the Sertoli cell secretory products are of protein nature. Probably well preserved morphology and function of Sertoli cell in vitro is due to extracellular matrix secreted by Sertoli cell (M a t h e r, P h y l i p s, 1984) which creates the base for the cell attachments. In addition we have established that SCCM-12 markedly stimulate proliferation of somatic BALB/c 3T3 cells. 7-fold increase of [^{3}H]-thimidine incorporation over controls was shown after application of 20 μg/ml protein. In comparison SCCM-6 stimulate markedly prespermatogonial proliferation up to 10-folds over controls.

Based on the above mentioned results we could speculate that in spent media Sertoli cells secrete a mitogenic factor(s) which effectively stimulate proliferation of quiescent prespermatogonia. Mitogenic activity of SCCM from 6th-day-old rats is more effective on prespermatogonial proliferations since at this day prespermatogonia in the testis begin to divide, and they need a stimulus for the mitosis.

At the 12th day p.p. germ cells enter meiosis and the mitogenic activity of SCCM proteins is decreased. The putative mitogenic factor(s) appears to be proteinaceous with mol.weight over 8000 Da and sensitive to heat and trypsin treatment (K a n - c h e v a et al., 1990).

Having in mind the effect of purified SGF on prespermatogonial cell proliferation (M a r t i n o v a et al., 1992) it could be one of the most probable growth factor secreted by Sertoli cells. The coctail of growth factors secreted by Sertoli cell consist of SCSGF, FGF, TGF, IL-1, and the balance between different growth factors depends on the stage of differentiation of germ cells as well as of the rest cell types in the testis. Our results are in agreement with the hypotesis that Seroli cells mediate the

action of pituitary hormones and secrete a number of growth factors which participate in control of germ cell proliferation.

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