Original Article

EFFECT OF FETAL CALF SERUM ON CELLULAR PROLIFERATION OF MOUSE Y1 ADRENOCORTICAL CELLS IN VITRO

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ABSTRACT

Objective: Fetal calf serum (FCS) is a supplement used in cell culture media for successful culturing. The present study was design to observe the effect of FCS on cellular proliferation.

Methodology: Effect of different concentrations (0, 1, 5, 10 and 20%v/v) of FCS on cellular proliferation was determined by the uptake of crystal violet, MTT assay. Total cellular protein was also measured colorimetrically to observe the effect of FCS on growth of mouse Y1 adrenocortical cells.

Results: The results showed a gradual increase in proliferation of Y1 cells by increasing concentrations of FCS in Dulbecco's modification of Eagle's medium (DMEM). Highest proliferation rate of Y1 adrenocortical cells was achieved with cultured cells after 6 days in DMEM medium containing 10 to 20% FCS.

Conclusion: The study suggested that supplementation of 20% FCS increase cell proliferation and acts as a growth factor results in cell division and DNA synthesis.

KEYWORDS: FCS, cell proliferation. Y1 adrenocortical cells.

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INTRODUCTION

Fetal calf serum (FCS) is a supplement used in many cell culture media for successful culturing.[1,2] It is known to contain growth factors and cytokines necessary for cell division.[3] Supplementation of FCS in developing embryos results in DNA synthesis and cell proliferation.[1,4-6]

Growth factor signalling involves the use of multiple intermediate signals including calcium mobilization, inositol-phosphate formation and cAMP/cGMP formation. The initial signal sent by the ligand-receptor complex is converted into more widespread and general effectors, which are capable of initiating the multiple intracellular changes needed for cellular division.[7-11] Addition of FCS to certain fibroblastic cells results in activation of DNA

synthesis by increasing the concentration of intracellular Ca^{++}. This observation clarifies the mechanism of growth activation.[6]

Addition of FCS to resting cultures cause immediate changes in transport rates and cyclic nucleotide concentrations, followed by enhancement of the rates of protein and RNA synthesis, and ultimately by a resumption of DNA replication. It has been suggested that the various changes in the response to serum are coordinate effects; however, since serum contains many different growth factors, and hormones, the various observable effects could be brought about by separate mechanisms.[6]

The aim of present study was to determine the effect of different concentrations of FCS at different time on proliferation of mouse Y1 adrenocortical cells.

METHODOLOGY

Cell Culture: Y1 cells were grown in triplicate culture plates by using Dulbecco's modification of Eagle's medium (DMEM) containing 1-20% fetal calf serum, 2mM glutamine, 100μg/ml penicillin and 100 mg/ml streptomycin.[6-16] Culture were maintained at $37C^0$ humidified atmospheres of 5% CO_2 and 95% air.

FCS treatment on cell culture: The cells were removed from their substratum using trypsine (0.05%)-EDTA (0.02%) and were plated out at a density of about 0.25×10^5 cells/well (prepared in DMEM medium without FCS) into the center 60 wells of two 96 well plates, counted using a haemocytometer. Cells were allowed to settle overnight in the humidified gas incubator and then treated with different concentrations (0, 1, 5, 10 and 20% v/v) of FCS, 12 wells/treatment used for growth.

Crystal violet stain: Crystal violet method of measuring cell proliferation relies upon its uptake by the cells, which eventually stains DNA. The intensity of color produced is proportional to cell number. For the uptake of crystal violet, cells were allowed to proliferate in response to stimulatory agent for 48h. At the end of experiment, cell media were removed and cells were washed with 200 ml PBS. Cells were then fixed with 200 ml of methanol for 15 minutes. Crystal violet (0.1% solution in 200 mM boric acid) was added as 200 ml/well for 20 minutes and proliferation was determined by the intensity of color produced on solubilising the dye in 50 ml 10% glacial acetic acid for 30 minutes in gas incubator. Color produced was measured at 540nm using a plate reading multi-scan spectrophotometer.[5]

MTT Assay: The logarithmic growth phase of Y1 adrenocortical cells were taken for growth assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenly tetrazolium bromide (MTT). The MTT assay relies on the uptake of MTT, a water-soluble tetrazolium salt producing a yellowish solution. In cell cultures it is converted to an insoluble blue formazan by cleavage of the tetrazolium ring by dehydrogenases of mitochondria of living cells. The intensity of the blue color produced is a measure of viable cell number.[5] For the MTT assay, cells were cultured with stimulatory agents for 48h and 20ml of MTT (5mg/ml solution in PBS) was added to each of the treated cultures 4h before the end of the experiment and cells were incubated at 37^0C in a humified 5% CO2 incubator. To achieve solubilization of formazan crystals formed in viable cells, DMSO was added to each well, and the absorbance was recorded on a micro titer plate reader at a test wavelength 570nm with a reference wavelength of 690 nm. The optical density (O.D) was calculated as a difference between the absorbance at the reference wavelength and that observed at the test wavelength. The effect of drugs on growth was assessed as percent of cell viability.[17]

Measurement of total cellular protein: Proliferation of Y1 adrenocortical cells was tested using a non-radioactive nucleotide analogue, 5-bromo-2'-deoxy-uridine (BrdU, Boehringer, Mannheim). Cells were seeded at a density of $10000/cm^2$ on flat-bottom 96 well micro titer plates in culture medium with or without 1 to 20% FCS, medium was changed every 48 hr. The cells were incubated for 16 hr with BrdU, rinsed twice with culture medium, fixed,

washed and stained with an anti-BrdU antibody linked to peroxidase. The peroxidase cleavage substrate was detected colorimetrically at 490 nm versus 405 nm (dual wavelength ratio). The results of the BrdU measurements are expressed in arbitrary units (ratio x 1000).[15]

Total cellular protein was determined after 20 min of cell cleavage with Triton X100 1%v/v using a phenol reagent micro protein method (Sigma, Munich). Standards were prepared by dilution of bovine serum albumin. All data are expressed as µg protein per single well. The number of cells per well was determined by counting cell nuclei on photomicrographs of methylene blue stained cells on a standardized portion of the well bottom. Statistical significance was calculated using Student's t-test, all data are given as arithmetic means±standard error.

RESULTS

Determination of cell proliferation by crystal violet stain: To asses the effect of FCS on cellular proliferation we studied the uptake of crystal violet by Y1 adrenocortical cells. The cells were grown as monolayer cultures and treated with different concentrations of FCS (0, 1, 5, 10 and 20% v/v) to stimulate cellular proliferation. The results showed that there was significant increase in proliferation of Y1 cells with the increase in amount of FCS in the DMEM medium. There was increase in absorbance value from 0.197 to 0.516 when concentration of FCS added in the medium increased from 0 to 20% (Table-Ia).

To further study the maximum effect of FCS dose on cell proliferation we studied the crystal violet method. The results showed that a sharp increase in absorbance of Y1 cells was observed when 1% of the FCS was added in the DMEM medium, flowed by a gradual increase up to the addition of 10% FCS, finally a lower rate of increase in absorbance was achieved when concentration of FCS in the medium raised from 10-20% (Fig-1).

Determination of cell proliferation by MTT assay: To further conform the cell proliferation effect by addition of FCS, we studied MTT assay. The results showed that there was a significant increase in absorbance value from 0.069 to 0.243 when concentration of FCS added in the medium from 0 to 20% (Table-Ib). There was a gradual increase in absorbance value up to the addition of 5% FCS in the medium followed by a sharp increase in absorbance by the addition of 10 and 20% FCS (Fig-2).
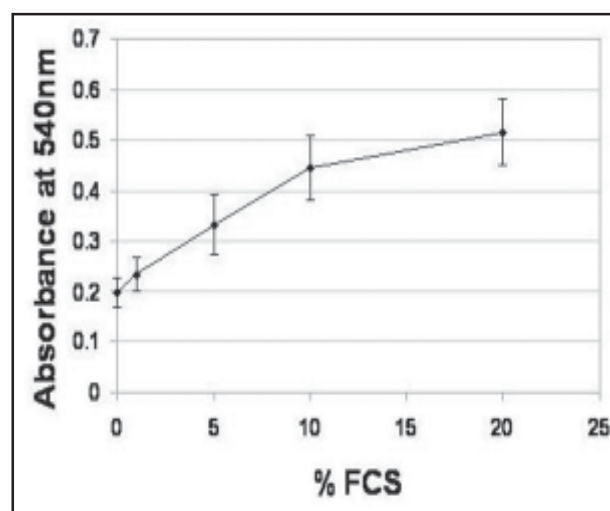

Fig-1: Graph showing the effect of different concentrations of fetal calf serum on proliferation of mouse Y1 adrenocortical cells using crystal violet staining. X-axis showing the percentage of fetal calf serum and Y-axis showing the absorbance at 540nm.
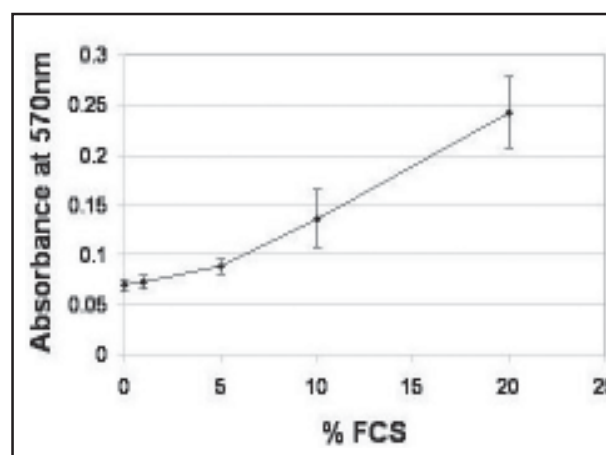

Fig-2: Graph showing the effect of different concentrations of fetal calf serum on proliferation of mouse Y1 adrenocortical cells using MTT assay method. X-axis showing the percentage of fetal calf serum and Y-axis showing the absorbance at 570nm.

Increase in total protein after FCS treatment To asses the increase in total protein after 20% v/v concentrations of FCS on cellular proliferation, that may be the result of either cell enlargement (hypertrophy), or of cell proliferation, we counted the cells after application of FCS at 2 and 6 days after plating. On day 2, total cell number without FCS ($3.10 \pm 0.15 \times 10^4$ cells/well) slightly differs from cells treated with 10% of FCS ($4.70 \pm 0.15 \times 10^4$ cells/well). On day 6, FCS treated cells numbered $5.991 \pm 0.19 \times 10^4$ cells/well, an increase compared to the $4.10 \pm 0.10 \times 10^4$ cells/well of the controls, cells treated with 10% of FCS numbered $5.50 \pm 0.15 \times 10^4$ cells/well which was significantly different from controls. Total protein after 20% of FCS treatment rose from 90 ± 6 to 150 ± 4 pg/cell from the second to sixth day after plating (n=3) (Fig-3).

To further analyze the time and different concentrations of FCS treatments. The effect was detected from 2 to 6 days, but was most pronounced at an incubation period of 6 days. At this time, total protein doubled on 20% FCS (9.28 ± 0.60 vs. 4.90 ± 0.54 µg/well). Total 10 and 20% FCS treatment showed its maximum effect on total protein incorporation as compare to control group (Fig-3).
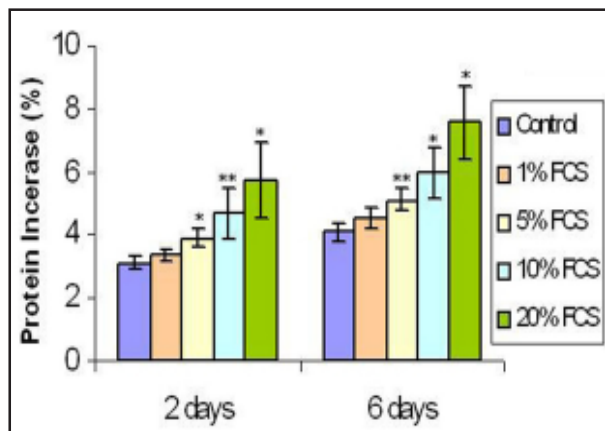

Fig-3: Dose and time dependent effect FCS on proliferation of Y1 adrenocortical cells The influence of different concentrations of FCS at varying time in culture concentration on Y1 adrenocortical cells. The vertical axis shows the relative increase of total protein content in a single well of a 96 well microtiter plate. A value of 100% is equivalent to the protein content at each given time in culture without the addition of FCS. Statistically significant differences in protein content between a treatment and control group *$p<0.05$, and **$p<0.01$. (n=3).

DISCUSSION

Proliferation capability and cell viability was quantitatively determined by the incorporation of crystal violet and MTT on living cells. In the present study, we observed that the crystal violet staining showed that adrenocortical cells actively proliferated with the addition of FCS under incubation of 1% of FCS resulting in a sharp increase in absorbance. Further, cells proliferation was confirmed by MTT assay, gradual increase in absorbance was found up to the addition of 5% FCS. This suggested that although proliferation rate was same as in crystal violet method, but there was less formation of blue formazan product due to the presence of less amount of mitochondrial dehydrogenase. However no reduction in increase in absorbance rate during 10 to 20% FCS addition observed in crystal violet and MTT assay (Fig-1 & 2).

An overall increase in absorbance by Y1 adrenocortical cells achieved due to the stimulation of cell proliferation by the addition of FCS in crystal violet, MTT assay and total cellular protein was also measured colorimetrically. Previously, Bertsch and Marks,[4] Langendonckt et al.,[9] and May et al.,[10] reported increased cellular proliferation with the addition of FCS in culture medium. Increased proliferation rate of adrenocortical cells by the addition of FCS occurred due to multiple effects because of the presence of epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) in FCS.[10,12,14] These multiple effects include calcium mobilization, inositol-phosphate formation and cAMP/cGMP formation due to growth factors signalling. The initial signal sent by the ligand-receptor complex is converted into more widespread and general effectors, which are capable of initiating the multiple intracellular changes needed for DNA replication and cellular division.[7] Finally, the results

indicated that FCS contained growth factors necessary for cell division and is a strong inducer of cell proliferation.

ACKNOWLEDGMENTS

REFERENCES

1. Cuchet D, Ferrera R, Lomonte P, Epstein1 AL. Characterization of antiproliferative and cytotoxic properties of the HSV-1 immediate-early ICP0 protein. J Gene Med 2005;7:1187–99.

2. Sagirkaya H, Yagmur M, Nur Z, Soylu MK. Replacement of fetal calf serum with synthetic serum substitute in the in vitro maturation medium: Effects on maturation, fertilization and subsequent development of cattle oocytes in vitro. Turk J Vet Anim Sci 2004;28:779-84.

3. Sasse M, Lengwinat T, Henklein P, Hlinak A, Schade R. Replacement of fetal calf serum in cell cultures by an egg yolk factor with cholecystokinin / gastrin-like immunoreactivity. ATLA 2000;28(6):815-31.

4. Gerharz CD, Gabbert HE, Biesalski HK, Engers R, Luley C. Fetal calf serum and retinoic acid affect proliferation and terminal differentiation of a rat rhabdomyosarcoma cell line (BA-HAN-1C). British J Cancer 1989;59(1):61-7.

5. Sallot M, Lascombe I, Bermont L, Jouvenot M. Comparison of the effects of epidermal growth factor and fetal calf serum in human endometrial carcinoma RL95-2 cells. Anticancer Research. 1997;17(5A):3499-504.

6. Bertsch S, Marks F. Effect of fetal calf serum and epidermal growth factor on DNA synthesis in explants of chick embryo epidermis. Nature 1974;251:517-9.

7. Dulbecco R, Elkington J. Induction of growth in resting fibroblastic cell cultures by Ca++. Proc Nat Acad Sci 1975;72(4):1584-8.

8. Gilman AG. G proteins and regulation of adenylyl Cyclase. In: Physiology or medicine, Department of Pharmacology, The University of Texas, USA 1994;182-212.

9. Jin-Song HE, Horikoshi S, Funabiki K, Shirato I, Tomino Y. Cerivastatin inhibits fetal calf serum-induced DNA synthesis in cultured rat mesangial cells. Nephrology 2002;7(2):83-6.

10. Langendonckt AV, Donnay I, Schuurbiers N, Auquier P, Carolan C, Massip A, et al. Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J Reproduction and Fertility 1997;109:87-93.

11. May JV, Bridge AJ, Gotcher ED, Gangrade BK. Regulation of porcine theta cell proliferation in vitro: Synergistic actions of epidermal growth factor and platelet-derived growth factor. Endocrinology 1992;131(2):689-97.

12. Rubin B, Cooley MA, Kaoue, LD, Taylor RB, Golstein P. Production and main characteristics of a fetal calf serum-specific cell line that induces T and B cell differentiation. Scandinavian J Immunology 1980;12:401-9.

13. Rudland PS, Gospodarowicz D, Seifert W. Role of fibroblast growth factor (FGF) in cell signalling and proliferation. Nature 1974;250:741-73.

14. Sagirkaya H, Misirlioglu M, Kaya A, First NL, Parrish JJ, Memili E. Developmental potential of bovine oocytes cultured in different maturation and culture conditions. Anim Reprod Sci 2007;101(3-4):225-40.

15. Schwegler JS, Knorz MC, Akkoyun I, Liesenhoff H. 1997; Basic, not acidic fibroblast growth factor stimulates proliferation of cultured human retinal pigment epithelial cells. Molecular Vision 1997;15:3:10.

16. Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-Ll adipocytes. J Biol Chem 1988;263(19):9402-8.

17. Wiesner DA, Dawson G. Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway. J Neurochem 1996;66:1418-25.