Title: Effects of Sheep Brain-Derived Factors on Primary Cultures of Kidney Cells

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Abstract:

Sheep and rabbit organs were collected and assessed for growth properties at the PPU Biotechnology Research Center's (BRC) animal cell culture unit. A combination of mechanical and enzymatic disaggregation techniques proved successful in generating cells of kidney origin that formed clean monolayers of confluent cells in culture. The growth characteristics of adult tissues were too slow to be practicably viable, but new-born organs generated cells capable of rapid growth. In order to identify the best conditions for culturing cells, a range of parameters were assayed and we report here that a moderate supply of soluble factors extracted at the BRC from a sheep's brain had a profoundly beneficial effect upon the growth characteristics of new-born sheep kidney cells.

Introduction:

The primary culture of animal cells is a necessary requirement for*in-vitro* testing of anti-cancer drugs, vaccines and infectious diseases of livestock. They have the advantage of being cheap to acquire, readily available and they better mimic in-vivo conditions than do commercially available cell culture lines. The difficulty with their use lies in standardisation of procedures and consistent supplies, as well as their requirement for a delicate mix of exogenous growth factors(Hoesli et al., 2012) that are very costly to purchase. Standardisation of protocols for locally supplied animal organs is currently a priority at the BRC and we report here the use of sheep brain-derived growth factors to supplement the growth of primary cell cultures at a fraction of the cost of using commercially available cell lines or growth factors. Previous studies had shown the mitogenic enhancing effect of pig brain-derived factors (Višnja et al., 2009), but we chose to utilize sheep brain because of the absence of locally available pig brain and, in addition, we predicted that sheep brain would give a species-specific advantage.

Methods:

Sheep and rabbit organs were transported to the BRC under aseptic conditions in phosphate buffered saline and the tissues were macerated with a sterile scalpel and treated with 0.25% trypsin-EDTA till disaggregation was observable. Cell suspensions were then plated on $25cm^2$ T-necked flasks with DMEM medium including 10% fetal

calf serum (Jayme and Blackman , 1985), and cultured in a humidified, 5% CO_2 incubator at 37^0C.

Sheep brain extracts were prepared by homogenizing a sheep brain with 200ml of phosphate buffered saline solution and the filtrate collected after course filtering using surgical gauze, followed by passing through a 0.22μm filter with a syringe to maintain sterility cell growth media and supplements. The filtrate was aliquoted and stored frozen at -20^0C till use.

Flasks were marked with equal sized circles (ϕ=0.4 cm, area=0.125cm^2) and the cells counted daily after plating to assess proliferation and survivability. Cells were counted using a phase contrast inverted light microscope at 100X magnification.

Results:

Cells grew to 100% confluency and formed a healthy monolayer (Fig 1a) when grown on 25cm^2 T-necked flasks with DMEM. The morphology of the cells appeared epithelial-like as characterized by the distinctive 'cobblestone' pattern that was 1 cell thick and indicated contact inhibition. Cell rounding and detachment after treatment with trypsin occurred readily after 15 minutes (Fig 1b) making these cells amenable to passage, which we were able to perform for three separate rounds of sub-culture without any apparent detriment to cell morphology or growth rate.

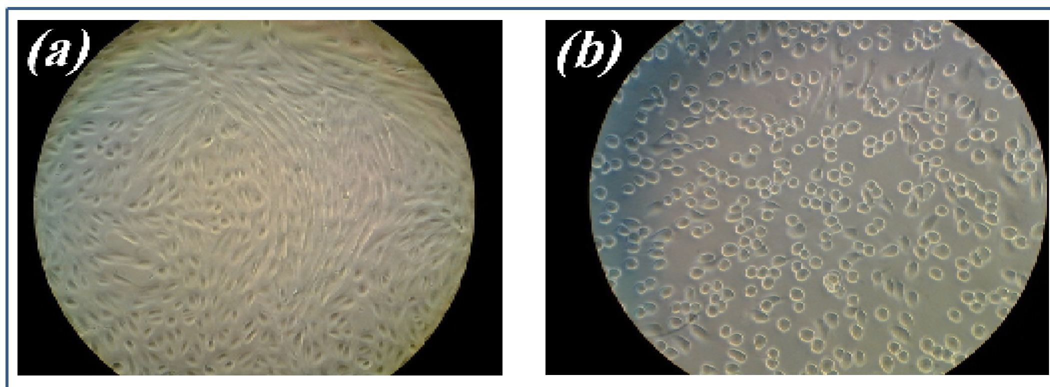

Figure 1. Confluent kidney cell monolayer at 100X magnification through a phase contrast inverted light microscope.

The growth curves for sheep kidney cells over 7 days (Fig 2) demonstrate a 2.8-fold increase in number for the flask of cells that was not supplemented with brain extract, while the presence of 100μl and 500μl of brain extract increased the cell number 4.2-fold and 4.3-fold respectively over the same time period. However, larger input volumes of brain extract resulted in a reduction in growth rate that fell below the basal rate of growth for cells cultured without extracts.

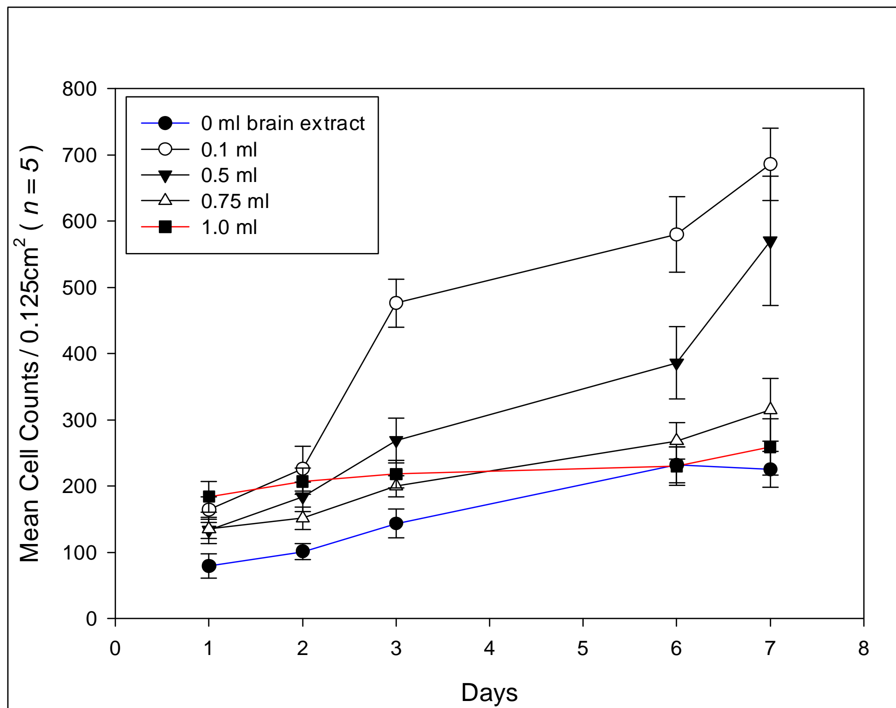

Figure 2. Growth curves for sheep kidney cells cultured in DMEM medium with various quantities of brain extracts in ml. Error bars are standard deviations, and n =5 replicates.

In order to assess the possibility that a non-specific, nutritional factor was responsible for enhanced growth with modest supplementation by sheep brain extract, or that a similarly non-specific, cytotoxic effect of the sheep brain extract was responsible for suppressing expansion of the cell population at higher concentrations, xeno-supplementation of rabbit kidney cells was performed with sheep brain extract (Fig 3).

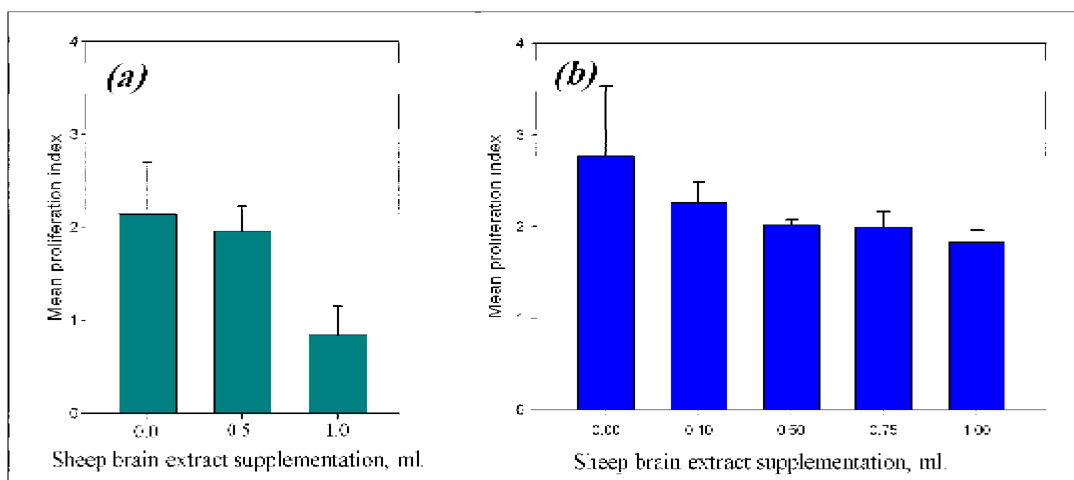

Figure 3. Effect of xeno-supplementation upon proliferation of rabbit kidney cells with sheep brain extract in (a) DMEM for 7 days and in (b) RPMI medium for 4 days. Error bars are standard deviations, and n=10 replicates were performed for the zero supplement controls and n=5 for the supplementation with sheep brain extracts.

It is apparent from Figure 3a that xeno-supplementation of rabbit kidney cells does not result in any increase in proliferation, in contrast to the sizeable boost given to sheep kidney cells (Fig 2), which indicates that the effect on sheep kidney cells is a species-specific effect and not an artefact. At the highest concentration, however, proliferation was retarded, which is indicative of a generalized cytotoxic effect at high concentrations of sheep brain supplement. Overall growth with 0.5ml of brain extract, of rabbit kidney cells was somewhat less than that of sheep kidney cells and reached only 1/6th the cell number observed for the sheep cells over parallel time frames. This may have been due to a special nutritional requirement of the rabbit cells that DMEM was unable to satisfy, and so the experiment was performed again using an alternative media, RPMI, and the results plotted in Figure 3b. This time, growth was so rapid that the cell counting was stopped after only 4 days as a confluent monolayer had been achieved. Xeno-supplementation confirmed the earlier observation in DMEM of a species-specific effect of low doses of sheep brain supplement, but this time failed to show a significant inhibition even at the highest dose of supplement.

After the success of growing rabbit kidney cells in RPMI medium, rabbit lung cell extracts were prepared and plated into25cm^2 T-necked flasks with the same medium in the absence of sheep brain supplements, but this time, no growth was supported and cell numbers rapidly declined(Fig 4). This may be due to lack of collagen coating on the culture flasks, which is needed as a substratum for the firm attachment of some sheep cell types (Radi and Ackermann, 2004). Adult sheep kidneys also proved to be an unsuitable source of cells as their growth curve was almost stationary (data not shown).

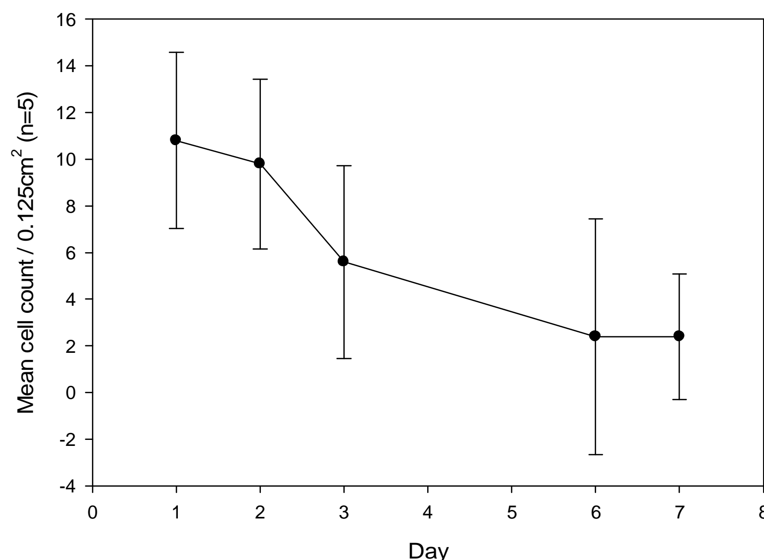

Figure 4. Senescence curve for rabbit lung cells in RPMI without supplementation. Error bars are standard deviations, and n = 5 replicates.

Conclusions:

Healthy monolayers of sheep kidney cells can be cultured using new born sheep kidneys with DMEM medium and supplementation with brain extracts. Input of higher quantities of brain extract had an inhibitory effect, indicating the presence of competing enhancing and inhibitory factors that interact depending upon different dosage rules to give an overall enhancement in growth rate at lower quantities and an overall inhibition at higher quantities. Use of the sheep brain extract for xeno-supplementation confirmed that the growth enhancing effects were species-specific while the inhibitory effects may be non-specific in nature. Ongoing work seeks to better understand this process and refine the optimal growth conditions in our primary cell culture system.

Acknowledgements and Author Contributions:

Mohammad Yahia Ismaiel Alswalhe is an M.Sc. student who is using animal cell culture for the study of animal disease and he is setting up a cell culture system for his assays. Mohammad Yousef Manaserah is a final semester M.Sc. student and veterinarian who supplied the sheep brain and also surgically resected the organs and prepared them under aseptic conditions, while Hassan Al-Taradeh gave valued technical support within the remit of the BRC animal cell culture facility. Robin Abu Ghazaleh is the supervisor of both students and is responsible for this manuscript. All authors have shared in reviewing the manuscript.

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