Milk Supplementation Effects on Fibroblastic Cells

Justin Beiriger
Central Catholic High School
Grade 11

Question

How does breast milk affect the proliferation of fibroblastic cells in an infant's body?

Purpose

The purpose of this experiment was to determine the effect of breast milk on 3T3 Fibroblastic cells

Breast Milk

- Produced in the mammary glands of a human female
- Primary source of nutrition for infants
  - Exclusive breastfeeding for the first six months of life
  - Supplemental breastfeeding until age 2
- Has many health benefits:
  - Increased intelligence
  - Lower risk of Sudden Infant Death Syndrome
  - Lower risk of ear infections
  - Lower risk of childhood leukemia
  - Lower risk of diabetes
  - Lower risk of asthma and eczema
  - Decreased dental problems
  - Decreased psychological problems

Breast Milk

- Hormones such as prolactin and oxytocin signal breast milk production
- Composition of breast milk varies day-to-day depending on diet, mood, sleep pattern, etc.
- Breast milk provides passive immunity for the child
  - He/she is not old enough to defend him/herself from pathogens
- Contains approximately 0.8% protein, 4.5% fat, 7.1% carbohydrates, and 0.2% minerals
- Has many antibacterial and healing properties

3T3 Cells

- Cell line established in 1962 by two scientists in New York
- Considered the standard fibroblastic cell line
- Obtained from mouse embryo tissue
- Do not differentiate
- Produce Extracellular Matrix (ECM) parts and structural proteins
- Often used as a model for human fibroblasts.

Materials

- **Cryotank**
- 75mm2 tissue culture treated flasks
- Six 25 mm2 tissue culture treated flasks
- Fetal bovine serum (FBS)
- **3T3 Fibroblastic Cell Line**
- Trypsin-EDTA
- Pen/strep
- Macropipette + sterile macropipette tips (1 mL, 5 mL, 10, mL, 20 mL)
- Micropipettes + sterile tips
- **DMEM Serum** - 1% and Complete Media (4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate + [10% fetal bovine serum for complete])

- **Human Breast Milk**
- 75 mL culture flask
- Incubator
- **Nikon Inverted Microscope**
- Aspirating Vacuum Line
- Laminar Flow Hood
- Laminar Flow Hood UV Sterilizing Lamp
- **Hemocytometer**
- Sterile PBS
- Ethanol (70% and 100%)

Procedure

- A 1 mL aliquot of 3T3 cells from a Cryotank was used to inoculate 30 mL of 10% serum DMEM media in a 75mm^2 culture flask yielding a cell density of approximately 10^6 to $2x10^6$ cells.
- The media was replaced with 15 mL of fresh media to remove cryo-freezing fluid and incubated (37° C, 5% CO_2) for 2 days until a cell density of approximately $4x10^6$ to $5x10^6$ cells/mL was reached.
- The culture was passed into 3 flasks in preparation for experiment and incubated for 2 days at 37°C, 5% CO_2.
- After trypsinization, cells from all of the flasks were pooled into 1 common 75mm^2 flask with a total volume of 10 mL.
- 1.0 mL of the cell suspension was added to six 25 mm^2 tissue culture treated flasks containing 5 mL of DMEM (com) media.

Procedure

- Two of the flasks were the control group, and were placed in the incubator for at least five hours to allow the cells to re-adhere.
- 0.01 mL of breast milk was added to two of the flasks so that they contain the low concentration (approximately 0.2%).
- 0.10 mL of breast milk was added to the remaining two flasks so that they contain the high concentration (approximately 2.0%).
- The flasks were incubated overnight in the same conditions as above.
- Each flask was trypsinized into a cell suspension.

Procedure

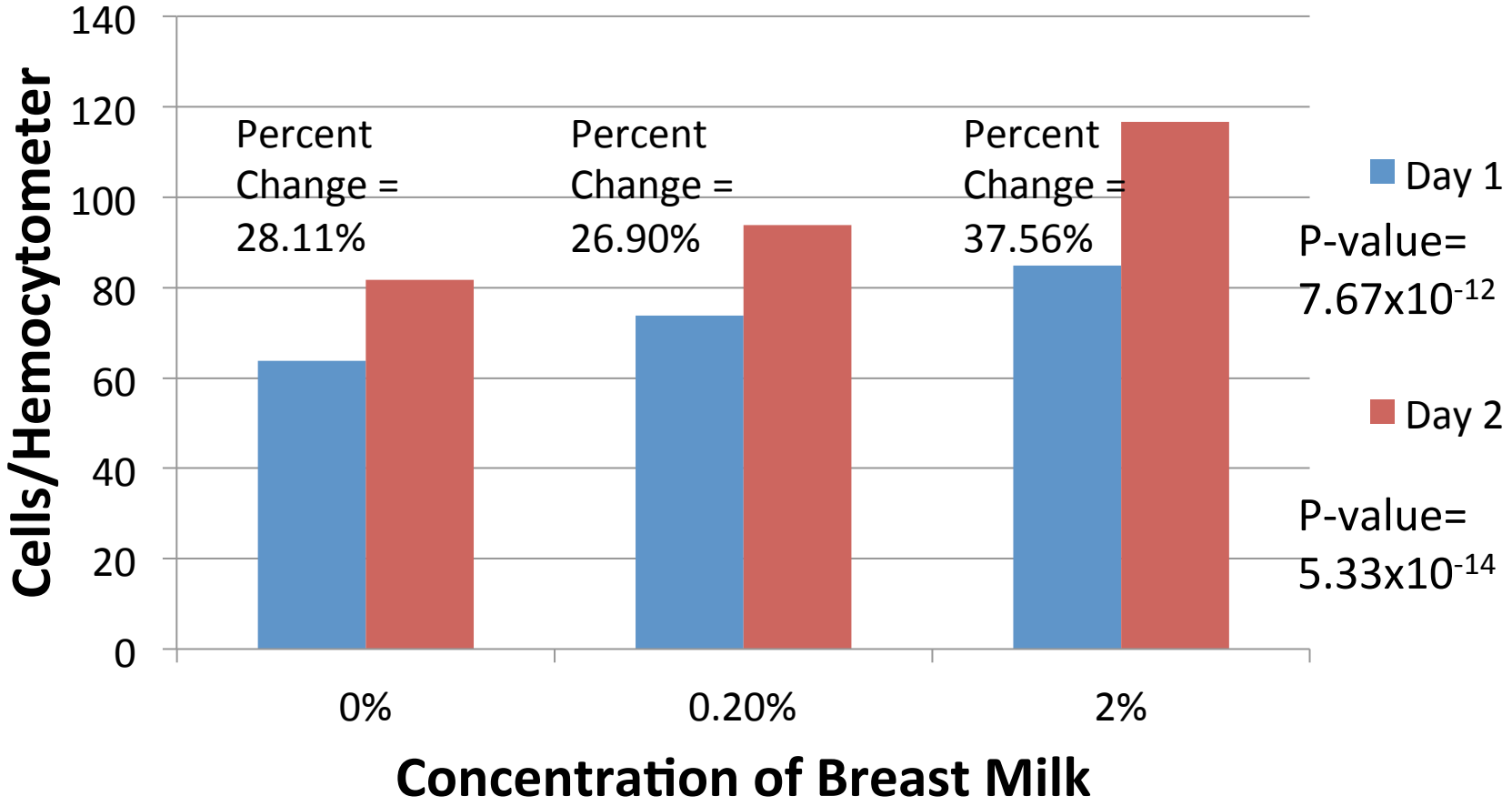
- The trypsin reaction was stopped with 5 mL of media to replace the previous amount and the appropriate amount of variable was added (same as previous exposure) into each flask.
- 25 µl aliquots were transferred to a Hemocytometer for quantification (eight counts per flask).
- The flasks were placed in the incubator overnight.
- Each flask was trypsynized into a cell suspension.
- 25 µl aliquots were transferred to a Hemocytometer for quantification (eight counts per flask).

Concentrations

	Control	Low Concentration	High Concentration
Media	5 mL	4.99 mL	4.90 mL
Breast Milk	0 mL	0.01 mL	0.10 mL
Total Volume	5 mL	5 mL	5 mL
Resulting Concentration	0%	0.02%	0.20%

Concentrations were constructed assuming infants consume approximately 100 mL of breast milk per sitting, and have a total of approximately 5 L of fluid in their bodies.

Proliferation Assay



ANOVA

- Abbreviation for analysis of variance
- Statistical test comparing variation within and between experimental groups

•If the P- value is lower than the alpha value (.05), then the result is significant (a result of the variable influence)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	1308	81.75	79.53333
Column 2	16	1500	93.75	43.13333
Column 3	16	1868	116.75	110.8667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10122.67	2	5061.333	65.01856	5.33E-14	3.204317
Within Groups	3503	45	77.84444			
Total	13625.67	47				

Sample ANOVA used in experiment

Dunnett's Test

$$t_d = \frac{M_i - M_c}{\sqrt{\frac{2MSE}{n_h}}}$$

- T-crit = 2.42 at alpha of 0.05
- T-value>T-crit indicates significance
- 0.02% breast milk exposure
  - Day 1: 4.66>2.42, Significant
  - Day 2: 9.76>2.42, Significant
- 0.2% breast milk exposure
  - Day 1: 3.85>2.42, Significant
  - Day 2: 11.22>2.42, Significant

Conclusion

- Statistical analyses strongly suggest significant variation due to 0.02% and 0.20% breast milk exposure after days 1 and 2
  - Breast milk significantly aided in the proliferation of fibroblastic cells
- Null hypothesis can be rejected
- Alternate hypothesis can be accepted

Limitations

- Hemocytometer counts may have varied
- Cell suspension was not homogeneous
- Pipetting was not perfectly synchronized
- Incomplete trypsynization
  - Not all cells were suspended
  - Low number of flasks

Extensions

- Wider range of concentrations
- Breast milk from mothers at different stages of breastfeeding
- Use other cell lines (C2C12, MG36)
- Test synergistic effects with other infant foods
- CyQUANT™ Cell proliferation assay
  - More quantitative than hemocytometer counts
  - Fluorescent dye binds to nucleic acid in cells

References

- http://www.nlm.nih.gov/medlineplus/ency/article/002451.htm
- http://www.breastmilk.com/
- http://www.americanpregnancy.org/firstyearoflife/whatsinbreastmilk.html
- http://en.wikipedia.org/wiki/3T3_cells
- http://nih3t3.com/
- http://www.sigmaaldrich.com/catalog/product/sigma/93061524?lang=en&region=US