

32k

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1 Aim

Investigate whether the growth of the melanoma cell line B16-F1-Luc2-BR2 is impaired in the presence of *beta*-hydroxybutyrate (BHB), over the 5 day period of the experiment.

We begin with 32×10^3 cells per 12-well plate.

2 Materials

- **B16-F1-Luc2-BR2** cells.

These were generated as follows:

- B16 cells were obtained from American Type Culture Collection (ATCC).

- To facilitate quantitative measurement of tumor growth, they were modified as described previously [1].

The cells were stably transfected with the gene encoding luc2 (luciferase) using the pGL4.51 [luc2/CMV/Neo] vector (Promega Corp, Madison, WI) and FuGENEH 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) following conditions specified by the manufacturer.

- They were then injected into the right ventricle of a mouse.

These animals were sacrificed when bioluminescence was detected in the brain.

- Cells metastatic to the brain were recovered put into culture.

- (R)-(-)-3-hydroxy butyric acid sodium salt (Sigma-Aldrich, St. Louis, MO).

- $2 \times$ 12-well plastic culture plates (Falcon ®)

- 12-well plastic culture plate (Falcon ®)

- Media: **DMEM** (Gibco ®) +10% FCS + 600 µg/mL G418 + 1x glutamine. pH 7.4.

FCS = Fetal calf serum.

- Trypsin/ EDTA.

- Hemocytometer (manual and automated (Countess™~by Invitrogen™)

- 15 mL plastic centrifuge tube (VWR™)

- Other supplies:
 - Trypan blue stain 10%.
 - Eppendorf tubes (plastic, sterile).
 - PCR tubes (for mixing cells and Trypan blue).
 - Disposable slides for hemocytometer.

3 Methods

3.1 Day 0

- The total cell count from the T-25 flask was 1104×10^3 .
- For the cells being grown with 10 $\mu\text{mol/L}$ BHB, add 0.016 39 g of (R)-(-)-3-hydroxy butyric acid sodium salt to the 50 mL tube before the media and cells.
- Seed 2×12 -well plates with 16×10^3 cells in 1 mL of media.
 For 16×10^3 cells, this is $\frac{16}{438} = 0.035$ mL.
 Add 13×0.035 mL = 0.46 mL to a 50 mL plastic tube (for mixing media with BHB) and top up to 13 mL.
 Mix well by pipetting up and down $\times 20$.
 Add 1 mL was to each of the 12 wells.
- Shake the plates gently to evenly distribute the cells.
 Return to the incubator at 17:30.
- Incubate at 37 °C, in 5% CO₂ for 24 h.

3.2 Days 1-4

- For plates which are not being counted:
 Aspirate the old media and replace with 1 mL of new media.
- For plates being grown in BHB, prepare the media was prepared by adding BHB as on day 0.
 The quantities of BHB required are shown in the table below.
- For plates to be counted:
 Add 2 mL of trypsin to each in the row to be counted.

Rock the plates.

Remove the trypsin after < 30 s.

- Add 250 μ L of trypsin.
- Incubate at 37°C for 3 min.
- Shake the plate to ensure all cells are dislodged from the base.
Check with the microscope.
- 'Quench' the trypsin quenched with 250 μ L of media.
Pipette up and down $\times 20$ using glass Pasteur pipette to ensure the cells are thoroughly mixed.
- Remove 11 μ L of cell suspension and add 11 μ L Trypan Blue in a sterile PCR tube.
Mix using a pipette.
- Place 10 μ L of the mixture into a sample well of the hemocytometer.
- Count the cells using the hemocytometer.
These counts are shown in table 3.
- Return plates to incubator.

Day	No. wells	Volume of media (mL)	BHB (grams)
1	9	10	0.01260
2	6	7	0.00882
3	3	4	0.00504

Table 1: Quantities of BHB required each day

3.3 Notes on methods

On day 3 observations were unable to be completed for reasons unrelated to the experiment.

This experiment was previously tried using initial seeding densities of 16×10^3 cells per mL. Using the same manual counting method, these cells appeared *much smaller* (< 0.5 diameter) and typically the fine-focus of the microscope was required to determine whether the cells were viable (i.e. had a visible halo and no sign of Trypan blue uptake).

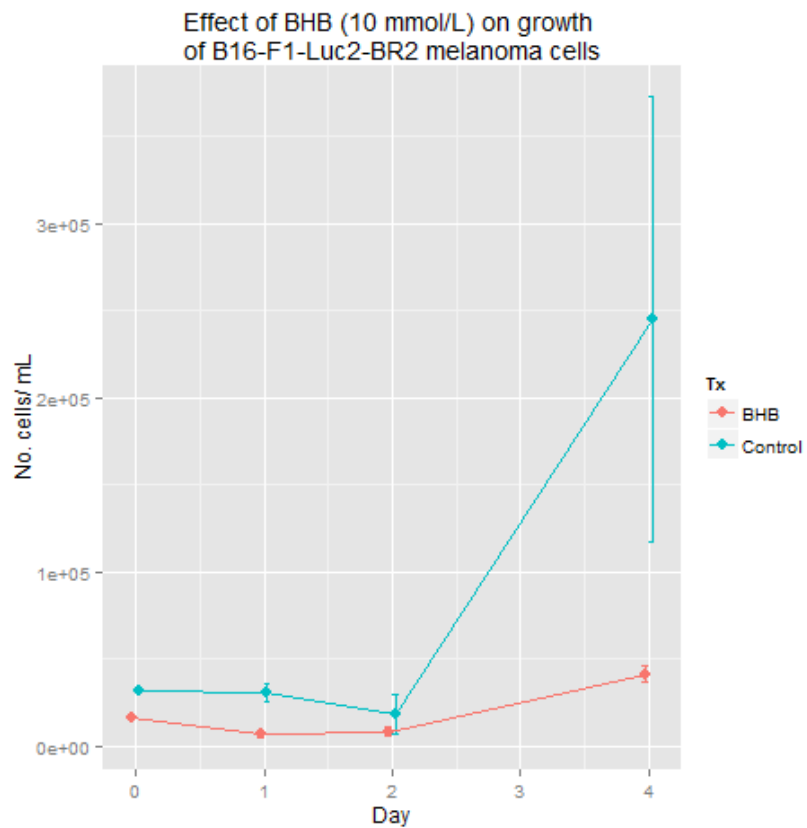
4 Results

4.1 Results

4.2 Standard error

```
#  $SE_{\bar{x}} = \frac{\sigma}{\sqrt{x}}$   
stdErr <- function(x) sqrt(var(x)) / sqrt(length(x))  
library(plyr)  
(df2 <- ddply(df1, c("Day", "Tx"), summarise,  
  mean = mean(Count),  
  SE = stdErr(Count)))
```

4.3 Plot



4.4 Day 4: t-test

```
with(df1[df1$Day==4, ], t.test(Count ~ Tx))
```

Welch Two Sample t-test

```
data: Count by Tx
t = -1.5915, df = 2.005, p-value = 0.2522
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -751654.7 344988.0
sample estimates:
    mean in group BHB mean in group Control
          41333.33          244666.67
```

4.5 Linear models: anova

```
l1 <- lm(Count ~ Day, data=df1)
l2 <- lm(Count ~ Day + Tx, data=df1)
print(xtable(anova(l1, l2),
  caption="Analysis of variance on linear models",
  align=c("l", rep("c", 6)),
  display=c("d", "d", "fg", "d", rep("fg", 3)),
  label="tab:data"),
  booktabs=TRUE)
```

	Day	Tx	Count
1	0	Control	32000
2	0	Control	32000
3	0	Control	32000
4	1	Control	20000
5	1	Control	36000
6	1	Control	36000
7	2	Control	42000
8	2	Control	8000
9	2	Control	6000
10	3	Control	128000
11	3	Control	
12	3	Control	
13	4	Control	492000
14	4	Control	66000
15	4	Control	176000
16	0	BHB	16000
17	0	BHB	16000
18	0	BHB	16000
19	1	BHB	12000
20	1	BHB	6000
21	1	BHB	4000
22	2	BHB	8000
23	2	BHB	14000
24	2	BHB	4000
25	3	BHB	
26	3	BHB	
27	3	BHB	
28	4	BHB	32000
29	4	BHB	46000
30	4	BHB	46000

Table 2: Cells per mL, day 0 - 4

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)
1	23	183900148148				
2	22	158550134759	1	25350013389	3.5	0.07406

Table 3: Analysis of variance on linear models

5 Conclusions

The cells grown with BHB seem to show impaired growth over time visually, although we could not confirm this formally on statistic testing ($p \approx 0.07$).

6 Appendix

6.1 Help on anova

Below, we give a printout from the standard R help for ANOVA for linear models.

```
print(help(anova.lm))
```

```
anova.lm                                package:stats                                R Documentation

_A_N_O_V_A _f_o_r _L_i_n_e_a_r _M_o_d_e_l _F_i_t_s

_D_e_s_c_r_i_p_t_i_o_n:

    Compute an analysis of variance table for one or more linear model
    fits.

_U_s_a_g_e:

    ## S3 method for class 'lm'
    anova(object, ...)

    anova.lmlist(object, ..., scale = 0, test = "F")

_A_r_g_u_m_e_n_t_s:

object, ...: objects of class 'lm', usually, a result of a call to
            'lm'.

test: a character string specifying the test statistic to be used.
      Can be one of '"F"', '"Chisq"' or '"Cp"', with partial
```

matching allowed, or 'NULL' for no test.

scale: numeric. An estimate of the noise variance σ^2 . If zero this will be estimated from the largest model considered.

`_D_e_t_a_i_l_s`:

Specifying a single object gives a sequential analysis of variance table for that fit. That is, the reductions in the residual sum of squares as each term of the formula is added in turn are given in as the rows of a table, plus the residual sum of squares.

The table will contain F statistics (and P values) comparing the mean square for the row to the residual mean square.

If more than one object is specified, the table has a row for the residual degrees of freedom and sum of squares for each model. For all but the first model, the change in degrees of freedom and sum of squares is also given. (This only make statistical sense if the models are nested.) It is conventional to list the models from smallest to largest, but this is up to the user.

Optionally the table can include test statistics. Normally the F statistic is most appropriate, which compares the mean square for a row to the residual sum of squares for the largest model considered. If 'scale' is specified chi-squared tests can be used. Mallows' Cp statistic is the residual sum of squares plus twice the estimate of σ^2 times the residual degrees of freedom.

`_V_a_l_u_e`:

An object of class '"anova"' inheriting from class '"data.frame"'.

`_W_a_r_n_i_n_g`:

The comparison between two or more models will only be valid if they are fitted to the same dataset. This may be a problem if

there are missing values and R's default of 'na.action = na.omit' is used, and 'anova.lmlist' will detect this with an error.

`_N_o_t_e:`

Versions of R prior to 1.2.0 based F tests on pairwise comparisons, and this behaviour can still be obtained by a direct call to 'anova.lm'.

`_R_e_f_e_r_e_n_c_e_s:`

Chambers, J. M. (1992) `_Linear models._` Chapter 4 of `_Statistical Models in S_` eds J. M. Chambers and T. J. Hastie, Wadsworth & Brooks/Cole.

`_S_e_e _A_l_s_o:`

The model fitting function 'lm', 'anova'.

'drop1' for so-called 'type II' anova where each term is dropped one at a time respecting their hierarchy.

`_E_x_a_m_p_l_e_s:`

```
## sequential table
fit <- lm(sr ~ ., data = LifeCycleSavings)
anova(fit)

## same effect via separate models
fit0 <- lm(sr ~ 1, data = LifeCycleSavings)
fit1 <- update(fit0, . ~ . + pop15)
fit2 <- update(fit1, . ~ . + pop75)
fit3 <- update(fit2, . ~ . + dpi)
fit4 <- update(fit3, . ~ . + ddpi)
anova(fit0, fit1, fit2, fit3, fit4, test = "F")

anova(fit4, fit2, fit0, test = "F") # unconventional order
```

References

- [1] Abdelwahab, Mohammed G., et al. The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. PloS one 7.5 (2012): e36197.