

16k2

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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 16×10^3 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 FuGENE 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.

- BHB (Sigma-Aldrich, St. Louis, MO).

- 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 zero

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The molecular weight of BHB is 126.

The calculations to convert to a target concentration of 10 mmol/l are shown in the set of equations beginning with 1.

$$1 \text{ mol} = 126 \text{ g} \quad (1)$$

$$10 \text{ mmol} = 1.26 \text{ g} \quad (2)$$

$$10 \text{ mmol/L} = 1.26 \text{ g/L} \quad (3)$$

$$= 0.00126 \text{ g/mL} \quad (4)$$

Thus 0.00126 g in 10 μ L added to 1 mL gives:

$$0.00126 \text{ g per 1 mL} = 10 \text{ mmol/l} \quad (5)$$

The quantities of BHB required each day are shown in table 1.

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: Quantity of BHB required by day

1. Cell counts from the T-25 flask are shown in table 2.

3.1.1 Cell counts on day zero

```
> df1 <- data.frame(Cells_per_mL=
+                   c(468e3, 344e3, 504e3))
> library(xtable)
> print(xtable(df1,
+               caption="Cell counts on day zero",
```

```

+           align=c("l", "c"),
+           display=c("d", "e"),
+           label="tab:ccd0"),
+       booktabs=TRUE)

```

	Cells_per_mL
1	4.68e+05
2	3.44e+05
3	5.04e+05

Table 2: Cell counts on day zero

3.1.2 Mean

```
> (m1 <- mean(df1$Cells_per_mL))
```

[1] 438666.7 The mean from the 3 counts was thus 438×10^3 mL.

- 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 $13 \times 0.035 \text{ mL} = 0.46 \text{ mL}$ 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% CO_2 for 24 h.

3.2 Days 1-4

1. For plates which are not being counted:
Aspirate the old media and replace with 1 mL of new media.
2. 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 table 1.
3. 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.
4. Add 250 μ L of trypsin.
5. Incubate at 37 °C for 3 min.
6. Shake the plate to ensure all cells are dislodged from the base.
Check with the microscope.
7. '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.
8. Remove 10 μ L of cell suspension and add 10 μ L Trypan Blue in a sterile PCR tube.
Mix using a pipette.
9. Place 10 μ L of the mixture into a sample well of the hemocytometer.
10. Count the cells using the hemocytometer.
These counts are shown in table 3.
11. Return plates to incubator.

4 Results

```
> df1 <- data.frame(Day = rep(rep(0:4, each=3), 2),
+                   Tx = rep(c("Control", "BHB"), each=15),
+                   Well = rep(LETTERS[1:3], 5),
```

```

+             Count = c(rep(16e3, 3),
+             32e3, 56e3, 6e3,
+             36e3, 14e3, 24e3,
+             90e3, 22e3, 18e3,
+             246e3, 38e3, 82e3,
+             rep(16e3, 3),
+             16e3, 34e3, 16e3,
+             20e3, 20e3, 24e3,
+             4e3, 12e3, 16e3,
+             20e3, 5e3, 12e3))
> print(xtable(df1,
+             caption="Cells per mL, day 0 - 4",
+             align=c("l", rep("c", 4)),
+             display=c("d", "d", "s", "s", "fg"),
+             label="tab:data"),
+       booktabs=TRUE)

```

4.1 Standard error

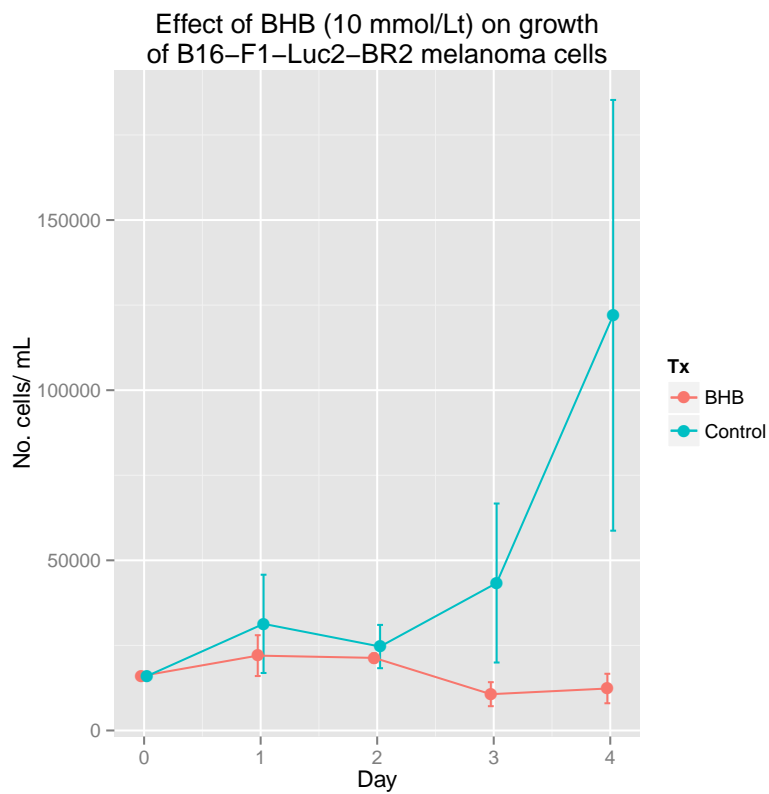
```

> stdErr <- function(x) sqrt(var(x)) / sqrt(length(x))
> library(plyr)
> ## summarise as mean and standard error
> ## to allow for plotting
> df2 <- ddply(df1, c("Day", "Tx"), summarise,
+             mean = mean(Count),
+             SE = stdErr(Count),
+             'mean+SE' = mean(Count) + stdErr(Count),
+             'mean-SE' = mean(Count) - stdErr(Count))
> print(xtable(df2,
+             caption="Mean counts, with standard errors",
+             align=c("l", rep("c", ncol(df2))),
+             display=c("d", rep("fg", ncol(df2))),
+             label="tab:means"),
+       booktabs=TRUE,
+       include.rownames=FALSE)

```

4.2 Plot

```
> library(ggplot2)
> ## plot with position dodge
> pd <- position_dodge(.1)
> ggplot(df2, aes(x=Day, y=mean, color=Tx)) +
+   geom_errorbar(aes(ymin=mean-SE, ymax=mean+SE),
+   width=.1, position=pd) +
+   geom_line(position=pd) +
+   geom_point(position=pd, size=3) +
+   ggtitle("Effect of BHB (10 mmol/Lt) on growth
+ of B16-F1-Luc2-BR2 melanoma cells") +
+   ylab("No. cells/ mL")
```



4.3 Day 4: t-test

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

Welch Two Sample t-test

```
data: Count by Tx
t = -1.7288, df = 2.019, p-value = 0.2248
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -380193.9 160860.6
sample estimates:
      mean in group BHB      mean in group Control
      12333.33              122000.00
```

4.4 Compare linear models

```
> ## linear models perhaps not justified here
> ## but this is the simplest choice
> l1 <- lm(Count ~ Day, data=df1)
> l2 <- lm(Count ~ Day + Tx, data=df1)
> a1 <- anova(l1, l2)
> print(xtable(a1,
+             caption="Analysis of variance \n when adding Tx to linear model o
+             align=c("l", rep("c", 6)),
+             display=c(rep("d", 2), "e", "d", "e", "fg", "fg"),
+             label="tab:anova"),
+       booktabs=TRUE)
```


	Day	Tx	Well	Count
1	0	Control	A	16000
2	0	Control	B	16000
3	0	Control	C	16000
4	1	Control	A	32000
5	1	Control	B	56000
6	1	Control	C	6000
7	2	Control	A	36000
8	2	Control	B	14000
9	2	Control	C	24000
10	3	Control	A	90000
11	3	Control	B	22000
12	3	Control	C	18000
13	4	Control	A	246000
14	4	Control	B	38000
15	4	Control	C	82000
16	0	BHB	A	16000
17	0	BHB	B	16000
18	0	BHB	C	16000
19	1	BHB	A	16000
20	1	BHB	B	34000
21	1	BHB	C	16000
22	2	BHB	A	20000
23	2	BHB	B	20000
24	2	BHB	C	24000
25	3	BHB	A	4000
26	3	BHB	B	12000
27	3	BHB	C	16000
28	4	BHB	A	20000
29	4	BHB	B	5000
30	4	BHB	C	12000

Table 3: Cells per mL, day 0 - 4

Day	Tx	mean	SE	mean+SE	mean-SE
0	BHB	16000	0	16000	16000
0	Control	16000	0	16000	16000
1	BHB	22000	6000	28000	16000
1	Control	31333	14438	45771	16896
2	BHB	21333	1333	22667	20000
2	Control	24667	6360	31026	18307
3	BHB	10667	3528	14194	7139
3	Control	43333	23362	66695	19971
4	BHB	12333	4333	16667	8000
4	Control	122000	63288	185288	58712

Table 4: Mean counts, with standard errors

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)
1	28	5.24e+10				
2	27	4.52e+10	1	7.21e+09	4.3	0.04772

Table 5: Analysis of variance when adding Tx to linear model of cells counts by day

5 Conclusions

The cells grown with BHB show signs of impaired growth vs. controls. Due to the large standard error on day 4, no difference could be demonstrated via a t-test.

However comparing linear models with and without treatment *does* show a significant effect ($p = 0.048$).

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.