Biostatistics 517 Autumn 2007 Group #2

Abstract

Backround

Difluoromethylornithine (DFMO) has previously been identified as a compound that may be useful in prevention of colon cancer and is postulated to act via reduction of polyamine levels. This phase IIb clinical trial evaluated the effect of DFMO on tissue levels of three polyamines in individuals with a history of colonic polyps.

Objectives

The study's primary objective was to assess the effect of DFMO on polyamine levels after 6 months and 1 year of treatment. The secondary objective was to determine whether DFMO affects polyamine levels 3 months after treatment, either rebounding to higher than baseline levels or remaining low. The effect of DFMO after 6 months of treatment and the effect of varying doses of DFMO were examined as exploratory objectives.

Methods

Patients were randomized to treatment with placebo or 0.075, 0.2 or 0.4 g/m^2/day, and were followed for 12 months of treatment and 3 months after treatment. Polyamine levels were measured at 0, 6, 12 and 15 months. The ratio of spermidine to spermine was chosen as an optimal measure of polyamine levels. Unpaired t-tests were used to compare this ratio between patients on each dose of DFMO and patients taking placebos. Treatment and placebo groups were compared 6 and 12 months into the treatment period and 3 months after treatment to assess the effect of DFMO relative to placebo. Participants in the highest and lowest dose groups were compared at 12 months to detect an effect of varying doses.

Results

The ratio of spermidine to spermine was found to be significantly reduced in the 0.075 g/m^2/day dose group at 6 months into treatment and in the 0.075 g/m^2/day and 0.4 g/m^2/day dose groups 12 months into treatment. No difference between treatment and placebo patient ratios was detected 3 months post-treatment. Similarly, no difference in ratio between high and low dose groups was found.

Conclusions

DFMO administration was associated with a reduction in polyamine levels during the treatment period, with the earliest statistically significant response detected 6 months into the treatment period. There was a non-significant trend toward higher levels post-treatment and appeared to be a small dose-response relationship in the doses tested. We recommend further investigation of this compound as a cancer prevention agent to be performed at the lowest two dose groups, with more in-depth evaluation for toxicity and an extended follow-up period to allow for detection of recurrent polyps and the development of colonic neoplasms.

Comment [A1]: How many?

Comment [A2]: which polyamines?

Comment [A3]: presumably the mean ratio

Comment [A4]: Estimates, estimates, estimates, estimates. Statistical significance on its own is irrelevant. It is merely a descriptor of the precision with which we know some scientific value.

We would want to know whether the mean difference was clinically important, as well as whether there were individual levels that might be toxic.

Comment [A5]: Problmes with missing data? To the extent that thes might be dose dependent, this might be worth putting into the abstract.

Comment [A6]: All polyamines, or just spermidine. Was the ratio lower because the numerator was lower or because the denominator got bigger.

Background and questions of interest

Among all malignancies, colon cancer is the third most common cancer and ranks second in the United States as a cause of cancer death. Factors that definitively protect against the development of colorectal cancer have not been identified, making this disease a key area for basic science research and pre-clinical and clinical trials.

Tumorigenesis is a complex process, influenced by genetics and environment, but ultimately requires abnormal growth and differentiation of cells. Thus, compounds that exert an effect on cellular

metabolism are key targets for anti-cancer drug development. One metabolic pathway relevant to colon cancer is that of polyamine synthesis. The diamine, putrescine and the polyamines, spermidine, and spermine, interact with vital cellular components, including nucleic acids, proteins, and the macromolecules that compose the cell membrane. Studies have shown that their levels are increased during periods of cell growth, so compounds to inhibit their synthesis have been developed as anti-cancer agents. One such agent is difluoromethylornithine (DFMO), a non-competitive, irreversible inhibitor of ornithine decarboxylase, the enzyme that mediates the first step in the set of biochemical reactions below. Importantly, metabolism of the amino acids arginine and methionine occurs through this pathway.[1]

Ornithine→ Putrescine→ Spermidine→ Spermine

DFMO causes cellular levels of putrescine and spermidine to decrease, but usually does not have an effect on spermine.[2] The reason for the maintenance of spermine concentrations is not fully understood, but may be related to recovery of putrescine and spermidine from the diet, overcoming the effect of DFMO.

Studies in the rodent model have found that DFMO administration both reduces tissue polyamine levels and inhibits growth of intestinal tissue.[3] In addition, a study in humans has shown that ornithine decarboxylase levels are increased in dysplastic human colonic polyp tissue relative to normal biopsies, suggesting that this pathway may be important in abnormal colonic growth and possibly involved in the development of colonic neoplasms.[4] DFMO provided uncertain benefit as a single agent for cancer treatment in a phase I-II trial, but its utility in cancer prevention has not been adequately explored.[5]

Supported by data from in vitro, animal studies, and phase I and IIa trials in humans, we have undertaken a phase IIb trial, to further investigate DFMO as an anti-cancer compound. In this randomized, double-blind, placebo-control study, we examined the effect of DFMO on polyamine levels in colon tissue, in order to determine the required dose, the rapidity of action, and the durability of its effects. Specifically, the primary aim was to assess the effect of DFMO on polyamine levels after 6 months and 1 year of treatment. The secondary aim was to determine whether DFMO affects polyamine levels 3 months after treatment, either rebounded to higher than baseline levels or remained low.

Source of the data

A Phase IIb clinical trial, to assess DFMO's effect on polyamine levels in colonic polyps, in order to explore its potential as a preventive agent for colon cancer was conducted on 114 adult men and women with a history of colon polyps. Subjects were randomized into one of four groups; placebo and one of three DFMO dose groups (0.075 g/m^2 per day, 0.20 g/m^2 per day, and 0.4 g/m^2 per day). The treatment period extended for 12 months, following. Measurements were conducted on each subject at baseline, during treatment administration (6 and 12 month follow-up), and 3 months following discontinuation of DFMO treatment (15 month follow-up). We measured levels of putrescine, spermine, and spermidine, from colon polyp tissue obtained by biopsy at all follow-up study visits.

Statistical Methods

Descriptive statistical techniques were used for each measurement to examine the distribution of data and quantify missingness. The relationships between sex and age and the ratio of spermidine to spermine were tested to assess for possible confounding. The effect of sex was tested with an unpaired, unequal variances t-test between ratio values in men and women. The effect of age was measured by Pearson correlation. Neither variable was a strong confounder, so no corrections were made.

Previous data has shown that putrescine and spermidine levels are affected by DFMO, but that spermidine levels tend to remain stable during treatment.[1] Putrescine is more precisely classifed as a diamine and cannot substitute for the required polyamines spermidine and spermine in cellular growth.[1] In addition, in our data set there were 26 undetectable measurements for putrescine, suggesting that the laboratory assay may not be optimized for detection of this molecule. Earlier studies have shown spermine levels to be relatively stable, and have suggested the ratio of spermidine to spermine as an optimal measurement of polyamine levels.[1,6] We adopted this approach. The distribution of ratios is highly skewed, but a log-transformation returns their distribution to a more normal shape with fewer outliers. Thus the natural log of the ratio of spermidine to spermine was chosen to be the primary summary measurement comparator, requiring comparisons of geometric rather than arithmetic means. Two-sample t-tests on the natural logs of the ratios yield inference about the ratio of geometric means of the spermidine/spermine ratios. P-values refer to the null hypothesis that the ratio of the geometric means is 1. Estimates and confidence intervals for the ratios of geometric means are reported.

For each dose group and in relevant time intervals, two-sample t-tests were run comparing the geometric means of patients on DFMO to patients taking a placebo. In the 6 and 12 month follow-up samples, this analysis tested whether treatment had an effect on polyamine levels. In the 15 month samples, this analysis tested for a prolonged effect of treatment or for a rebound to worse than normal polyamine levels. A 2-sample t-test was also run on patients on the highest and lowest nonzero doses after 12 months of treatment. This analysis tested whether the different doses had different effects: assuming a monotonic dose-response relationship, differential effects of dose would be clearest in the comparison between the extreme doses (and more sophisticated analyses are beyond the scope of our current statistical repertoire). All analyses were performed using R (version 2.6.1, Free Software Foundation, Inc.).

Results

Subjects

Our trial included 114 participants, of whom 85% (n=97) were male and 15% (n=17) were female. Ages ranged from 45 to 81 years old. The mean ages of men and women in the study were similar (63.6 and 63.4 years, respectively). Furthermore, the age distribution was not significantly different by dose groups or by sex within dose group (data not shown). Age and sex were determined not to be significant confounders: the correlation between age and ratio was only 0.25, which we considered too insignificant to correct for, and a t-test between ratio values in men and women was not significant.

Polyamines

Table 1 presents the descriptive statistics of polyamine levels at each dose and follow-up time. One trend in missing data should be noted. 106 participants were measured 6 months into treatment. Another 9 participants dropped out before finishing the 12-month treatment and an additional 3 missed the follow-up measurement 3 month after treatment. These missing measurements could confound our

Comment [A7]: Hopefully you would have chosen your model prior to looking at the data, and all of this discussion is just about whether you did further exploratory analyses.

Comment [A8]: For what it is worth, the use of the ratio is motivated somewhat by the variability in taking a biopsy (varying amounts of colon mucosa versus stromal tissue) and laboratory variability: The ratio constitutes some sort of an internal control on these two factors.

Comment [A9]: Again, this should be based on knowledge from before looking at the data. Whenever we look at ratios we do tend to use logs.

Comment [A10]: run on log transformed data (you said it above—I would say it again just for emphasis)

Comment [A11]: The degree of confounding would depend upon the correlation between age and dose and the magnitude of the slope parameter (not the correlation) between age and the log ratio after adjustment for dose. So your criterion for assessing confounding is faulty.

You cannot test for confounding. Period.

And you should not do data-driven hypothesis testing.

Comment [A12]: This table nicely includes min and max so we could assess very low or very high values—important from a toxicity standpoint, even though I am not sure of a particular value that would be deemed toxic.

conclusions: it is possible participants were lost to follow-up due clinically relevant reasons such as improvement in health status, cancer-related mortality, or adverse effects of the drug. More participants in the highest dose group dropped out (35%) than in any other. This large loss of participants may confound our results.

Comment [A13]: Maybe, maybe not. (I do admit that I would wonder if subjects having a lower ratio led to the dropout) But even more important would be whether this was a sign of toxicity (whether or not toxicity is due to the effect of DFMO on the spd:spm ratio or due to its effect on some other aspect.

Treatment Results

Table 2 shows the results of the t-tests comparing geometric means of spermidine/spermine ratios in the placebo group and different treatment groups at different time points. The primary analysis, a comparison of the geometric means during treatment at 6 months and at 12 months separately showed significant decreases in ratio at only some doses, and not the highest ones.

Comment [A14]: So comment on the patterns of the estimates: What was the driving force in the ratio going down, the numerator or the denominator.? Also, what were the trends in the estimates.? Is the lack of statistical significance driven more by the variability of the data or the lack of a monotonic trend in the data.

At the 6 month follow-up, we note that only the dose group of 0.075 g/m^2 per day had a significant decrease in the geometric means of spermidine/spermine ratio when compared to the placebo group. The ratio of geometric means (treatment:placebo) for dose group 0.075 g/m^2 per day is 0.726, which is typical of what we expect if the true ratio of geometric means were between 0.58 and 0.91. This translates to a 27.4% decrease compared to placebo (95% CI: 9% decrease to 42% decrease). Based on the two-sided P of 0.0055 from the t-test allowing unequal variances on the log transformed data, we can reject the null hypothesis that the ratio of the geometric means is 1. For dose group 0.2 g/m^2 per day, the ratio of geometric means is 0.745, typical of what we expect if the true ratio of geometric means were between 0.55 and 1.00. For dose group 0.4 g/m^2 per day, we see that the ratio of the geometric means is 0.754, which is not atypical of what we would expect if the true mean were between 0.54 and 1.06. Neither of these dose groups showed statistical significance as evidenced by the inclusion of 1 in their corresponding 95% confidence intervals or P values > 0.05 based on two-sided t-tests allowing unequal variances for the log transformed data.

At 12 month follow-up visit, there appears to be significant decrease for the spermidine/spermine ratio in the 0.075 g/m^2 per day and 0.4 g/m^2 per day dose groups. Similarly, the ratio of geometric means for dose group 0.075 g/m^2 per day is 0.728, which is typical of what we expect if the true ratio of geometric means were between 0.57 and 0.93. This translates to a 27.2% decrease when compared to placebo (95% CI: 7% decrease to a 43% decrease). Based on the two-sided P of 0.0118 from the t-test allowing unequal variances on the log transformed data, we can reject the null hypothesis that the ratio of the geometric means is 1. For dose group 0.4 g/m^2 per day, the geometric means of spermidine/spermine ratio is 0.663 (33.7% decrease compared to placebo), which is typical of what we expect if the true ratio of geometric means was between 0.50 and 0.88. Gathered from the two-sided P of 0.0052, based on the t-test allowing unequal variances on the log transformed data, we can reject the null hypothesis. For dose group 0.2 g/m2 per day, the geometric means of spermidine/spermine ratio is 0.672, which is not atypical of what we would expect if the true ratio of geometric means was between 0.45 and 1.01. The two-sided P of 0.0554, allowing unequal variance on the log transformed data, does not provide significant evidence to reject the null hypothesis.

Comment [A15]: I would have presented the results in order of dose, noting the trend toward lower ratios with increasing dose. Variability and sample size obviously also affect the statistical significance.

Duration of Response

To test for a prolonged effect of treatment or for a rebound to higher than normal polyamine levels, we examined the 15 month study visit for all dose groups (see Table 2). For dose group 0.075 g/m^2 per day, the geometric means of spermidine/spermine ratio is 1.14, which is typical of what we expect if the true ratio of geometric means was between 0.90 and 1.44, and a two-sided P of 0.2726 allowing unequal variances on the log transformed data. For dose group 0.2 g/m2 per day, the geometric means of

spermidine/spermine ratio is 1.310, which is not atypical if the true ratio of geometric means was between 0.97 and 1.77, and a two-sided P of 0.0759 allowing unequal variances on the log transformed data. For dose group 0.4 g/m^2 per day, the geometric means of spermidine/spermine ratio is 1.013, which is typical of what we expect if the true ratio of geometric means was between 0.82 and 1.25, and a two-sided P of 0.9057 allowing unequal variances on the log transformed data. In summary, no significant changes were observed in the geometric means of spermidine/spermine ratio in any of the groups.

However, Figure 1 shows a trend toward levels of spermidine/spermine greater than seen in untreated individuals occurring after the treatment period. In fact, the increase in the spermidine/spermine ratio may begin after the 6 month study visit. As shown in the figure, the ratio measurement in all dose groups is higher at the 12 month visit, with a rebound to a higher level following completion of the 12 month treatment. Future investigations should focus on a more extensive study to determine if the treatment truly has limited duration of response.

Comment [A16]: Be careful with this sort of analysis. We are always comparing across dose groups. We care more about how the comparison to the placebo group behaves, because we randomized across dose. Our measurements across time are confounded with aging, calendar time, and laboratory drift.

The comparison between dose group and placebo was generally lower at 12 mos than 6 mos.

Dose Response Effects

To conduct a simplistic analysis of dose effect on polyamine levels, we compared the geometric means of spermidine/spermine ratio in the 0.075 g/m^2 per day and 0.4 g/m^2 per day dose group. Table 3 presents the results of this analysis. We did not see a significant difference in the response of treatment with these two doses. The ratio of the geometric means of low to high dose groups (0.075 g/m^2 per day:0.40 g/m^2 per day) is 1.161, which is not atypical if the true ratio of geometric means were between 0.926 to 1.456. A two-sided P of 0.188, based on the t-test allowing unequal variances on the log transformed data, does not provide significant evidence to reject the null hypothesis. Therefore, we cannot conclude that there is an advantage to treating individuals with colon polyps with a higher dose of DFMO.

Comment [A17]: What time period?

Exploratory Analysis

Benefits did not seem to increase with a longer course of treatment, as polyamine levels did not differ significantly between the 6 and 12 month study visits in any dose group using a two sample t-test (data not shown).

Comment [A18]: Actually, you provided enough data to get the gist of this comparison. You seem to be living and dying by statistical significance. I think that is not wise. I tend to look at the trends as well. I see a trend toward lower values at 12 mos that I would have quantified with an estimate, and then I would have pointed out that we lacked precision to rule out all meaningful differences.

Discussion

We have performed a randomized, double-blind, placebo-controlled phase IIb trial of DFMO in individuals with colonic polyps in order to examine its effect on polyamine levels in colonic tissue. Our aims were to determine the doses that reduce polyamine levels, the length of treatment required, and the period of time over which the reduction in polyamines persists after discontinuation of the drug, in exploration of the use of DFMO as a cancer prevention agent.

We found that treatment with DFMO significantly reduces tissue polyamine levels, most notably in the 0.075 g/m^2/day dose group at 6 months, and in the 0.075 and 0.4 g/m^2/day doses at 12 months. This shows that the doses included in the study are biologically relevant in their ability to inhibit the ornithine decarboxylase enzyme, thereby affecting polyamine levels.

Comment [A19]: does this make scientific sense to you? Can you comment further?

To determine if DFMO's effect on polyamine levels is maintained after treatment has been discontinued, we looked at the spermidine/spermine ratio 3 months after the end of the treatment period. This analysis

revealed that levels did not remain low. In contrast, there was a tendency for polyamine levels to increase above placebo group levels, by more than 30% in the 0.2 g/m^2/day dose group, for example, but this did not achieve statistical significance. With any therapeutic agent, the goal is to find the minimum dose and duration of exposure at which it exerts the desired effect. Therefore, we recommend that future studies focus on lower doses of DFMO for two reasons. First, there was a statistically significant reduction in polyamine levels at the 0.075 g/m^2/day dose at 12 months. In addition, our data carries a suggestion of increased toxicity at the 0.4 g/m^2/day dose, evident by the larger number of missing measurements at the 12 and 15 month time-points in this group. Conversely, the missing data could also be the result of an improvement in health status in the highest dose group, leading to reduced motivation to participate in the study. However, we did not collect detailed information from participants regarding study dropout, so we cannot draw any definitive conclusions.

Comment [A20]: good

In summary, DFMO shows promise as a preventive agent for colon cancer, but more study will be needed to determine which doses will maximize efficacy and minimize toxicity. To this end, we recommend that a future study evaluate a larger number of participants using doses of 0.075 and 0.2 g/m^2/day and include objective measures to evaluate for toxicity as well as surveys of participant-reported adverse effects. Finally, since the development of cancer typically stems from multiple environmental and genetic effects over a period of years, long-term follow-up of participants will be required to determine if DFMO's effect on polyamines will translate into a reduction in polyp recurrence and tumorigenesis.

References

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TABLE 1: DESCRIPTIVE STATISTICS FOR PATIENT SPECIFIC MEAN POLYAMINE LEVELS BY DOSE GROUP FOR EACH MEASUREMENT, N = 117

Time (month)	Missing	Mean	SD	Min	Median	Max	Missing	Mean	SD	Min	Median	Max
	Dose Group 0 (nmol/mg protein, n=32)						Dose Group 0.075 (nmol/mg protein, n=29)					
Putrescine 0	0	0.66	0.44	0.06	0.57	1.98	0	0.65	0.52	0.01	0.54	2.59
6	2	1.06	1.59	1.05	0.72	9.14	1	0.47	0.27	0.00	0.49	1.05
12	4	1.16	0.83	0.00	0.90	3.18	3	1.08	1.03	0.04	0.69	4.29
15	5	1.54	1.77	0.30	0.80	6.51	3	1.19	0.89	0.00	0.91	3.53
Spermidine 0	0	3.26	1.45	1.40	2.93	7.05	0	3.47	1.55	1.51	2.91	7.02
6	2	3.37	1.53	1.51	3.02	6.91	1	2.64	0.89	1.39	2.46	5.12
12	4	3.26	1.31	1.01	2.82	5.91	3	2.92	0.99	1.35	2.86	4.92
15	5	2.69	0.93	1.25	2.45	4.62	3	2.95	0.99	0.00	2.98	4.83
Spermine 0	0	8.22	5.54	1.46	7.52	35.55	0	8.43	5.86	4.13	7.32	37.67
6	2	7.34	2.71	3.31	6.51	14.39	1	8.07	2.49	4.60	8.01	15.67
12	4	6.55	3.59	2.32	5.24	14.55	3	7.75	3.12	3.15	8.35	14.13
15	5	6.39	2.45	2.83	5.79	12.05	3	6.69	3.32	0.00	6.29	12.38
	Dose Group 0.2 (n=25)						Dose Group 0.4 (n=28)					
Putrescine 0	0	0.61	0.42	0.00	0.60	1.96	0	0.65	0.57	0.00	0.60	2.30
6	2	0.45	0.54	0.00	0.27	2.43	3	0.33	0.43	0.00	0.19	1.73
12	4	0.80	0.79	0.00	0.51	3.21	8	0.88	1.42	0.00	0.40	5.48
15	4	1.00	0.71	0.00	0.87	2.59	10	1.18	0.83	0.23	0.85	3.20
Spermidine 0	0	3.35	1.33	1.70	2.92	6.22	0	3.47	1.55	1.51	3.08	7.02
6	2	2.58	1.64	1.07	1.85	7.84	3	2.64	0.89	1.39	2.07	5.12
12	4	2.71	1.40	0.29	2.51	6.45	8	2.92	0.99	1.35	1.93	4.92
15	4	2.98	0.90	1.81	2.81	4.81	10	2.95	0.99	0.00	2.69	4.83
Spermine 0	0	9.03	7.04	2.54	7.53	41.68	0	8.43	5.86	4.13	6.81	37.67
6	2	7.18	2.48	2.35	7.37	12.13	3	8.07	2.49	4.60	8.01	15.67
12	4	7.16	3.15	2.96	6.63	13.83	8	7.75	3.12	3.15	5.97	14.13
15	4	6.08	3.56	1.93	4.61	12.38	10	6.69	3.32	0.00	6.00	12.38

TABLE 2: Comparison of geometric means of spermidine/spermine ratios in treatment and control groups

Dose Groups (gm/m^2 per day)	**Treatment Geometric Mean Spd/Spm Ratio**	**Placebo Geometric Mean Spd/Spm Ratio**	**Ratio of Geometric Means (Treatment:Placebo)**	**95% Confidence Interval of ratio of geometric means**	**P-value†**
	During Treatment at 6 month follow-up				
Dose Group 0.075	*0.324*	*0.446*	*0.726*	0.58, 0.91	*0.0055**
Dose Group 0.2	*0.333*	*0.446*	*0.745*	0.55, 1.00	*0.0515*
Dose Group 0.4	*0.336*	*0.446*	*0.754*	0.54, 1.06	*0.0972*
	During Treatment at 12 month follow-up				
Dose Group 0.075	*0.386*	*0.530*	*0.728*	0.57, 0.93	*0.0118**
Dose Group 0.2	*0.357*	*0.530*	*0.672*	0.45, 1.01	*0.0554*
Dose Group 0.4	*0.352*	*0.530*	*0.663*	0.50, 0.88	*0.0052**
	Post Treatment (15 month follow-up)				
Dose Group 0.075	*0.486*	*0.426*	*1.140*	0.90, 1.44	*0.2726*
Dose Group 0.2	*0.558*	*0.426*	*1.310*	0.97, 1.77	*0.0759*
Dose Group 0.4	*0.432*	*0.426*	*1.013*	0.82, 1.25	*0.9057*

† p-value based on two-sample t-test

* Indicates statistical significant at 0.05 level

Comment [A21]: I would include sample sizes here, because they varied by treatment group.

A more typical presentation would be to not give a column for the placebo, but instead to present the data as a separate row. Your way is not incorrect, however.

Figure 1. Mean ratios of spermidine to spermine

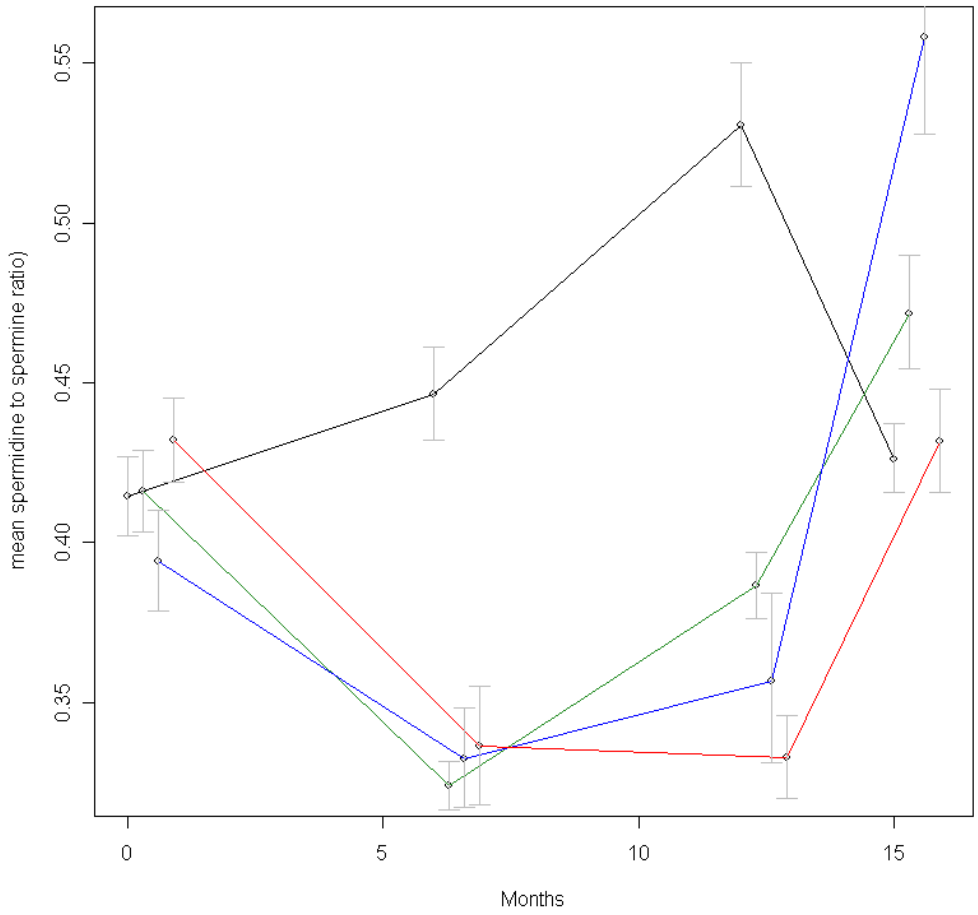

TABLE 3: Test for difference in geometric means of spermidine/spermine ratios during treatment in .075 and .4 dose groups.

	Low Dose (0.075) Geometric Mean Spd/Spm Ratio	High Dose (0.4) Geometric Mean Spd/Spm Ratio	Ratio of Geometric Means (low:high)	95% Confidence Interval of ratio of geometric means	P-value†
During treatment at 12 month follow-up	*0.386*	*0.333*	*1.161*	0.926, 1.456	*0.188*

† p-value based on two-sample t-test

* Indicates statistical significant at 0.05 level