TAUERET LABORATORIES

Study: Gene Expression Profile Changes Resulting from Ingestion of ASEA Redox Dietary Supplement: An Exploratory Study.
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Report Review

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Study overview:
This IRB approved observational study was constructed as a double blind, placebo controlled study designed to determine if gene expression profiles change over an 8-week period as a result of ingesting the ASEA Redox beverage. Blood samples were collected from 60 participants at two different time points: time 0 and 8 weeks. After 8 weeks post study, where participants did not consume ASEA Redox, participants in the test group were invited back for blood draws. The participants were randomized into test (group A), placebo (group B) or control group (group C). Participants completed a health questionnaire and a "symptoms" log over the course of the study. The study demographics reflected a 41% male, 59% female ratio, and had a mean age of 35. The study participant pool consisted of 92% Caucasian. RNA was extracted from the blood samples, gene expression levels tested and differential expression within and between groups analyzed.

Methodology and approach:
Peripheral blood samples were collected at time 0, 8 weeks, and again at 8 weeks following completion of the study in the ASEA Redox cohort. Total RNA was extracted from each sample using a PreAnalytix PAXgene Blood RNA Kit. After RNA extraction, each sample was concentrated by precipitation for globin RNA reduction using the Thermo Fisher GLOBINclear kit to prevent interference from excessive globin

transcripts. Globin reduced samples were then processed with the Affymetrix GeneChip 3' IVT PLUS kit followed by hybridization to an Affymetrix PrimeView Array that contains 49,395 probe sets across the human genome. The array was analyzed and files generated (.cel, .chp, .arr) to allow analysis of differential expression between time points. Quality control assessment was evaluated using the Affymetrix Expression Console software v 1.4 Jan 2017 and changes in expression levels were evaluated using the Affymetrix Transcriptome Analysis software v 3.0. Genes showing a change in expression over baseline in the test group (group A), and not seen in the placebo group (group B) or in the control group (group C) were identified as potential genes of interest. Additionally, differential expression was examined between group A week 8 and group B week 8 samples. Genes of interest were further examined for possible pathway connections using two web applications: Wikipathways and PANTHER. Finally, genes of interest were examined for expression levels at 8 weeks post-consumption of the ASEA Redox in the test group (washout experiment).

Results:

RNA quality:

The RNA quality was determined after final preparation for those samples selected for analysis. RNA concentrations and yields of the samples were determined by UV spectrophotometry (Nanodrop 1000, ND-1000 software v 3.8.1). An estimate of purity was determined with UV spectrophotometry by measuring the A260/A280 absorbance ratios. Additionally samples were analyzed on an Agilent 2100 Bioanalyzer using the 2100 expert software (vB.02.07.S153) to estimate integrity by examining the entire electrophoretic trace of the RNA. An estimated RNA Integrity Number (RIN) was generated. RIN numbers of greater than 6 are suggestive of RNA with integrity likely to work in downstream expression analysis. All samples had sufficient yield, 260/280 ratios and estimated RNA integrity numbers to suggest they were suitable for expression analysis. No samples were eliminated from the downstream analysis.

Post-array processing quality:

Using the Expression Console software a log probe cell intensity box plot was generated to further assess quality. Divergent probe intensity distributions relative to the other arrays may indicate that a sample should be eliminated from the analysis. Probe intensity was generally similar across samples at all time points implying minimal stratification from the experiments. From this analysis, one participant RNA was omitted from subsequent transcriptional abundance comparisons. This poor performance may be due either to slightly poorer quality of RNA or due to poor hybridization to the array.

Additional QC analysis of sample performance was completed with the Expression Console software. The microarrays contain hydridization, labeling and housekeeping gene controls. Samples not passing manufacture recommended thresholds for multiple controls were eliminated from the analysis.

Expression Analysis:

Expression analysis of time 0 (b2) vs. week 8 (wk8):

A total of 49372 probes were analyzed for statistical differences ($p<0.05$) between the placebo and ASEA Redox groups. Because there appeared to be little to no change in the expression data when held to a 2X fold change and corrected p-value threshold and not occurring in the other groups, we looked to find genes that had a nominal p-value of $p<0.05$ that demonstrated consistent change compared with the placebo group. ANOVA unpaired analysis of the placebo and the ASEA Redox groups with a nominal p-value of <0.05 was completed to identify potentially differentially expressed genes. Each group had 24 samples available for analysis. This analysis revealed 11 probes sets (11 genes) that met these criteria (Table 1 bolded). All probes in those genes were then examined for trends (Table 1).

For the 11 probes identified as differentially expressed, the fold change was considered in the ASEA Redox vs. Placebo and in Placebo time 0 vs Placebo at week 8 to identify that the difference observed was due to changes in the test group (Table 2). These values yielded fold changes of +/- 0.2-0.3, representing a 20-30% change in transcription.

Table 1. 11 genes (highlighted) showing differential expression in group A (ASEA Redox) test samples week 8 versus group B placebo week 8 samples plus all other probes in those genes.

Gene Symbol	Fold Change (linear) (A_WK8 vs. B_WK8)	ANOVA p-value (A_WK8 vs. B_WK8)
KCTD12	-1.06	0.323863
KCTD12	-1.21	0.103912
KCTD12	**-1.21**	**0.039723**
KCTD12	-1.06	0.315231
KCTD12	-1.08	0.311953
DNAJC3	-1.05	0.508502
DNAJC3	**-1.2**	**0.013674**
DNAJC3	-1.08	0.180221
EGR1	**1.22**	**0.00051**
EGR1	1.04	0.184945
EGR1	-1.05	0.540634
EMB	**-1.25**	**0.049527**
PYROXD1	**-1.31**	**0.044862**
PYROXD1	-1.11	0.267704
WDR11	**-1.2**	**0.026049**
IRAK3	-1.2	0.054293
IRAK3	**-1.2**	**0.016879**
IRAK3	**-1.28**	**0.020403**
CCR10	**1.2**	**0.003348**
CCDC126	-1.05	0.14749

CCDC126	**-1.22**	**0.008698**
CCDC126	-1	0.590658
IRAK3	-1.09	0.419533
PYROXD1	-1.05	0.077593
PYROXD1	1.02	0.747469
WDR11	-1.04	0.434389
EGR1	1.07	0.358741
EGR1	1	0.583142
EGR1	1.02	0.852381
IGLV1-41; IGLV1-51	**1.29**	**0.03319**
IGLV1-41	1.19	0.015031

Table 2. 11 probes differentially expressed in ASEA Redox test samples week-8 versus Placebo week-8 samples compared to Time 0 in the ASEA Redox and Placebo groups.

Gene Symbol	Fold Change ASEA Redox vs. Placebo	Fold Change (linear) ASEA Redox Time 0 vs. Week 8	Fold Change (linear) Placebo Time 0 vs. Week 8
KCTD12	**-1.21**	**-1.17**	**1.1**
DNAJC3	-1.2	-1.21	-1.11
EGR1	**1.22**	**1.4**	**1**
EMB	-1.25	-1.21	-1.15
PYROXD1	**-1.31**	**-1.25**	**1.03**
WDR11	-1.2	-1.11	-1.1
IRAK3	-1.2	-1.26	-1.06
IRAK3	**-1.28**	**-1.14**	**1.01**
CCR10	**1.2**	**1.19**	**1.02**
CCDC126	-1.22	-1.32	-1.09
IGLV1-41; IGLV1-51	1.29	1.34	1.22

There were 5 probes (Table 2 bolded) where the fold change from Time 0 to week 8 in the Placebo group was less than +/-1.05 (5%), suggesting that the 20-31% fold change observed in the week 8 ASEA Redox group was due to differences in the change from time 0. An additional probe in the IRAK3 gene was near this threshold. These gene/probe combinations were not differentially expressed in an environmental control group (taking no supplement or placebo) although the sample size of environmental control group may be too small to truly evaluate this.

Interestingly it was observed after an 8 week washout period the observed transcriptional changes were no longer maintained in the ASEA Redox group (Table 3).

Table 3. Expression analysis comparing ASEA Redox transcription activity in 5 genes of interest at week 8 and comparison of the transcriptional change comparing week 8 with the post study washout of 8 weeks with the ASEA Redox transcription at conclusion of study with participants consuming ASEA Redox.

Gene Symbol	Fold Change (linear) ASEA Redox 8 weeks vs. Time 0	Fold Change (linear) ASEA Redox Post study washout vs. Time 0
KCTD12	-1.17	1.44
EGR1	1.4	-1.02
PYROXD1	-1.25	1.74
IRAK3	-1.14	1.53
CCR10	1.19	-1.43

Expression analysis of the ASEA Redox week 8 sample vs. the ASEA Redox Post Study Washout sample showed the same trend as the ASEA Redox week 8 versus Time 0 sample (Table 4). Differential expression is trending in the same direction as the week 8 versus baseline data.

Table 4. Expression analysis comparing the fold change in transcription activity in 5 genes of interest at week 8 of the study consuming ASEA Redox with Time 0 and the change in activity comparing week 8 of the study with the conclusion of an 8 week washout period.

Gene Symbol	Fold Change (linear) ASEA Redox 8 weeks vs. Time 0	Fold Change (linear) ASEA Redox 8 weeks vs. ASEA Redox Post study washout
KCTD12	-1.17	-1.67
EGR1	1.4	1.39
PYROXD1	-1.25	-2.02
IRAK3	-1.14	-1.67
CCR10	1.19	1.54

The differential expression data of this follow up experiment suggest that the effect of the ASEA Redox beverage is not present at >8 weeks after stopping ingestion of the beverage. This can be concluded by the data, post versus baseline, trending in the opposite direction to the previously observed week 8 versus baseline data. In all five probe sets examined, those that had shown up regulation showed down regulation in the post versus baseline comparison, and samples that had shown down regulation showed up regulation in the post versus baseline comparison. Additional support for this finding is evident in the comparison of the week 8 versus post data. In this data the five probe sets all trended in a similar fashion as the week 8 versus baseline data. If the

probe set was down regulated in the original week 8 versus baseline data it was also down regulated in the week 8 versus post data and conversely if the original data was up regulated then the week 8 versus post data was also up regulated.
Confirmation of these findings may be further substantiated by a longer study time with larger cohorts of participants.

Gene and Pathway Information:
PANTHER (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships) is a classification system designed to classify proteins (and their genes) in order to understand gene pathways using high-throughput analysis. Using Panther Pathway Analysis (v11.1) the five genes of interest had five pathway hits. The five pathway hits involved three genes, CCR10, EGR1 and IRAK3 (Table 5).
Another pathway analysis software, Wikipathways, was also used to look for pathways associated with the genes of interest. Analysis revealed similar pathways for the genes identified in the Panther analysis.

Table 5. Panther Pathway Analysis

Gene	Pathway 1	Pathway 2	Pathway 3
CCR10	Inflammation mediated by chemokine and cytokine signaling pathway		
EGR1	Angiotensin II-stimulated signaling through G proteins and beta-arrestin	CCKR signaling map	Gonadotropin-releasing hormone receptor pathway
IRAK3	Toll receptor signaling pathway		
KCTD12	None		
PYROXD1	None		

Table 6. WikiPathways Analysis

Gene	Pathway 1	Pathway 2	Pathway 3	Pathway 4	Pathway 5
CCR10	Peptide GPCRs	GPCRs, Class A Rhodopsin-like	Chemokine signaling pathway	GPCR ligand binding	GPCR downstream signaling
EGR1	Serotonin Receptor 4/6/7 and NR3C Signaling	Brain-Derived Neurotrophic Factor (BDNF) signaling pathway	Circadian rhythm related genes	NRF2 pathway	VEGFA-VEGFR2 Signaling Pathway
IRAK3	Interleukin-1 signaling pathway	Regulation of toll-like receptor signaling pathway	MyD88:Mal cascade initiated on plasma membrane		
KCTD12	None				
PYROXD1	None				

Conclusions:

The primary analysis did not reveal any significant +/- 2-fold changes with significant p-values. Additional analysis did identify at least 5 genes that may have interesting differential expression in the test group and were not significant in placebo or control groups, when comparing the baseline expression to the week-8 expression. Pathway analysis revealed several gene pathways that could be further investigated.

Confirmation of these findings may be further substantiated by a longer study time with larger cohorts of participants. Additionally, the collection and expression analysis of the ASEA Redox subjects at 8 weeks or beyond from when the supplement consumption was stopped, provides interesting data on the length of time the supplement might have effects. These data suggest that continued use of ASEA Redox supplement is required to sustain continued transcriptional profile modulation.