The Toxicogenetic Effects of a Chitosan Based Hemostatic Control Patch in Rat Models

By

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I understand that my project will become part of the permanent collection of Oregon State University, Bioresource Research. My signature below authorizes the release of my project to any reader upon request.

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The toxicogenetic effects of a chitosan based hemostatic control patch in rat models

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Short title

Toxicogenetic effects of chitosan patches in rats

Abstract

Chitosan based hemorrhage control dressings provide effective external hemorrhage control while having few side effects. Known residual contaminants present in chitosan formulations include endotoxins and proteins known to stimulate gene expression in the liver. This study investigated gene expression in the liver using rat bio-compatibility models to gain knowledge regarding toxicogenetic effects caused by dressing contaminants. Gene chip technology was employed to evaluate effects of subcutaneous dressings on RNA expression in the liver. Various formulations of dressings containing known concentrations of endotoxin and control were evaluated over 8 or 28 days. Comparisons of cohorts suggest no correlation between endotoxin levels in dressings and hepatic gene expression. Additional studies are underway to test correlations between residual protein in dressings and gene expression.

Key Words

Chitosan

Endotoxin

Microarray

Rat

Introduction

Chitosan dressings have been developed as hemostatic dressings for number of clinical and emergency situations (Gustafson *et al.* 2007). They are widely deployed in Iraq and Afghanistan for trauma related bleeding and have recently been approved for non-military use (Becker 2003; Brown *et al.* 2007; Wedmore *et al.* 2006). Chitosans often contain varying levels of contaminants that include endotoxins and proteins (Dornish *et al.* 2001). Bio-absorbable formulations of chitosan based dressings are being considered for surgical use (Xie *et al.* 2008). A clear understanding of the humoral responses to chitosan formulations and the impact of the known contaminants are critical to the long term development of these products. Implantation studies in rats were conducted with the aim of characterizing *in vivo* responses to chitosan following subcutaneous placement of bandage material with known levels of endotoxin contamination.

The body processes toxic and immune responses differently and each pathway elicits distinct patterns of gene expression (Waring *et al.* 2006). Because of the unique toxicological responses, gene expression profiling would be expected to produce patterns of expression indicating a specific response related to the particular contaminant in the biomaterial.

In the present study we used microarray gene profiling technology to compare gene expression in the liver from animals exposed to various levels endotoxin through implanted chitosan dressings. Microarrays allow visualization of gene expression in various cell types. DNA microarrays use oligonucleotide primers to hybridize cDNA taken from a tissue sample, in our study of the liver. The Affymetrix Gene chips we used can have up to 16000-100000 different probes. Our working hypothesis was that endotoxin contamination in dressings would

result in changes in gene expression in the liver and indicate systemic endotoxin exposure. Following the gene expression analysis protein analysis was preformed to determine protein contamination.

Materials and Methods

The patches were provided by Hemcon Medical Technologies Inc. (Lake Oswego, OR), endotoxin levels were determined by Hemcon Inc. Rats were divided into test groups according the endotoxin level of the patch with which they were implanted. The endotoxin levels were 20 EU (endotoxin unit), 100 EU, 500 EU, 1070 EU and 1700 EU. A control group consisted of rats that underwent surgery but were not implanted with a patch. One group of exposed rats were euthanized and necropsied eight days after surgery and the other was euthanized and necropsied after 28 days. During necropsy a portion of the liver was removed. Total RNA was extracted from the set of liver samples in each of the test and control groups using a Qiagen RNeasy Micro kit (Qiagen, Valencia, CA) according to the manufacture's instructions with the following changes. The liver sample and 500 µl RLT buffer were homogenized using a dounce homogenizer. Five hundred µl ethanol was added to the solution, an equal volume was used if less supernatant was produced, and this new solution was mixed by pipeting. To ensure no buffer was carried over the column was centrifuged for one minute. Elutions of 48 µl were made, 25 µl sample was used to quantify total RNA. Total RNA was quantified on a ThermoFisher NanoDrop ND-1000 UV-Vis Spectrophotometer (ThermoFisher, Waltham, MA) using 2 µl. Nano-drop enables highly accurate analyses of samples as small as 1 µl. Biotin-labeled antisense RNA (target cRNA) was generated from total RNA using protocols recommended by the DNA microarray manufacturer (Affymetrix, Santa Clara, CA). Twenty µl

of cRNA preparation was fragmented and assessed on an Agilent Bioanalyzer 2100 for quality control.

Affymetrix analysis:

Samples were hybridized to an identical lot of Affymetrix GeneChip (RU34 A) for 16 hr. The Affymetrix GeneChip® system consists of a GeneChip® Scanner 3000, Affymetrix GeneChip® Fluidics Station 450, and Affymetrix GeneChip® Hybridization Oven 640.

Gene expression analysis was determined using the software R and Bioconductor (Vardhanabhuti *et al.* 2006) to generate Significance Analysis of Microarrays (SAM). Parameters tested included analysis of two-class unpaired case assuming unequal variances. Delta is the threshold level set for each analysis. As delta decreases, a greater number is gene products fall outside the threshold level. The cost of decreasing delta is an increase in the probability that the variance in gene expression is due to chance. Output included values for deltas of 0.1 to 1.3 as threshold levels for each analysis to estimate false discovery rate. As delta decreases, the ratio of "False" to "Called" genes increases and the False Discovery Rate (FDR) becomes greater.

The number of samples tested in each comparison presented was dependent on the minimum number of samples of sufficient quality in each cohort. If, for example, only 3 control samples were available for analysis in one group, then, three samples were chosen randomly from the Affymetrix cell files for that group to use as comparators.

Protein analysis

Each of the chitosan formulations used in the study was solublized by placing 10 mg of patch material into 0.5ml of RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Samples were heated to 60 ºC to increase the solublization of chitosan and 14 µl of the solution was loaded onto a 4-12% Bis-Tris Gel, 1mm

x 12 wells and electrophoresed for 30 minutes. Gels were duplicated so that one gel could be stained with NOVEX Stainer A, Colloidal Blue Stain and photographed. A second gel was electroblotted onto a nitrocellulose membrane. Nitrocellulose membranes containing chitosan and associated proteins were blocked with 2% dry milk in 1x TBE buffer for 1 hour at 25ºC followed by addition of 10 µl of serum from rats that demonstrated significant gross pathology from the 28 day chitosan implantation. The serum was collected at the time of terminal surgery. After 1 hour, blot was washed with two changes of wash buffer (1 x TBE with .1 % Triton X100). Blots were then incubated with a solution containing blocking buffer with goat anti-rat secondary antibody conjugated to alkaline phosphatase for detection. Autoradiographs of the blots were produced to visualize specific binding of serum antibodies to proteins contained within the chitosan.

Results

During the study some of the rats exhibited various physical responses. These symptoms included alopecia, skin lesions, and joint swelling. The physical symptoms subsided after about 14 days in most cases and occurred in all the patch formulations except the 20 EU group.

We used Significance Analysis of Microarrays (SAM) to determine whether gene expression changed in response to treatment with chitosan containing various levels of endotoxin. Figure 1 shows "called" genes, the number of genes whose expression deviated from the control, verses "false" genes whose change in expression was due to chance alone.

Liver gene expression in response to chitosan formulations with endotoxin levels of 100 EU (3 samples), 500 EU (5 samples) and 1070 EU (3 samples) did not differ significantly from surgical controls (Figure 1 & Figure 2). The 100 EU and the 1070 EU results were identical,

suggesting that endotoxin levels did not affect gene expression. However, at the highest level of endotoxin, 1700 EU (4 samples), when the threshold value Delta was set to 0.8, nine genes fell outside the range of expected expression (Figure 2). These genes are listed in table 1. However at Delta=0.8 the number of false positives increases, as stated earlier.

Protein Analysis

The presence of protein contaminants could be seen clearly in the columns of 1070 EU and 1700 EU patch samples (Figure 3). Little or no protein can be seen in the columns for the 100 EU and 500 EU test groups.

Discussion

The most significant findings in this study were seen in a comparison between the control animals and the chitosan patch formulation designated 1700. As can be seen in Figure 2-D, a small subset of genes falls away from the line for the 1700 EU. Figure 2 was generated using a delta of 0.8 which allows for a false discovery rate of 0.229. Under these conditions there are a number of gene products that are underexpressed compared to control rat livers. However, the genes do not appear to be related to each other nor do they represent a typical group of genes responsive to endotoxin (Table 1). For example, if the liver were responding to endotoxin we would expect the expression of a number of cytokines. The underexpression of these gene products is likely due to the expected false discovery rate rather than a response to treatment.

The results of comparisons at the 100 EU, 500 EU and 1070 EU levels were negative and no gene products were identified that suggest a change in gene expression. The 100 EU and the 1070 EU groups have identical results in gene expression. If the observed response were caused by endotoxin then the expression rates would be different. Because the expression rates are the

same, it is likely that other contaminants are responsible for the immune response and gene expression. It is more likely that contaminants other than endotoxin cause the observed immune response.

The protein analysis used rat serum from rats previously exposed to the chitosan patches. It is possible that the rat had a higher level of antibody response and this is reflected in the radiograph.

The results suggest that there is a numerical relationship between the level of endotoxin and the amount of a particular contaminant present, however because the patch formulations were all different this cannot be determined from this study alone. These contaminates may be due to manufacturing or processing. Further research and studies are needed to elucidate the factors causing immune response.

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Work sited

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Figures

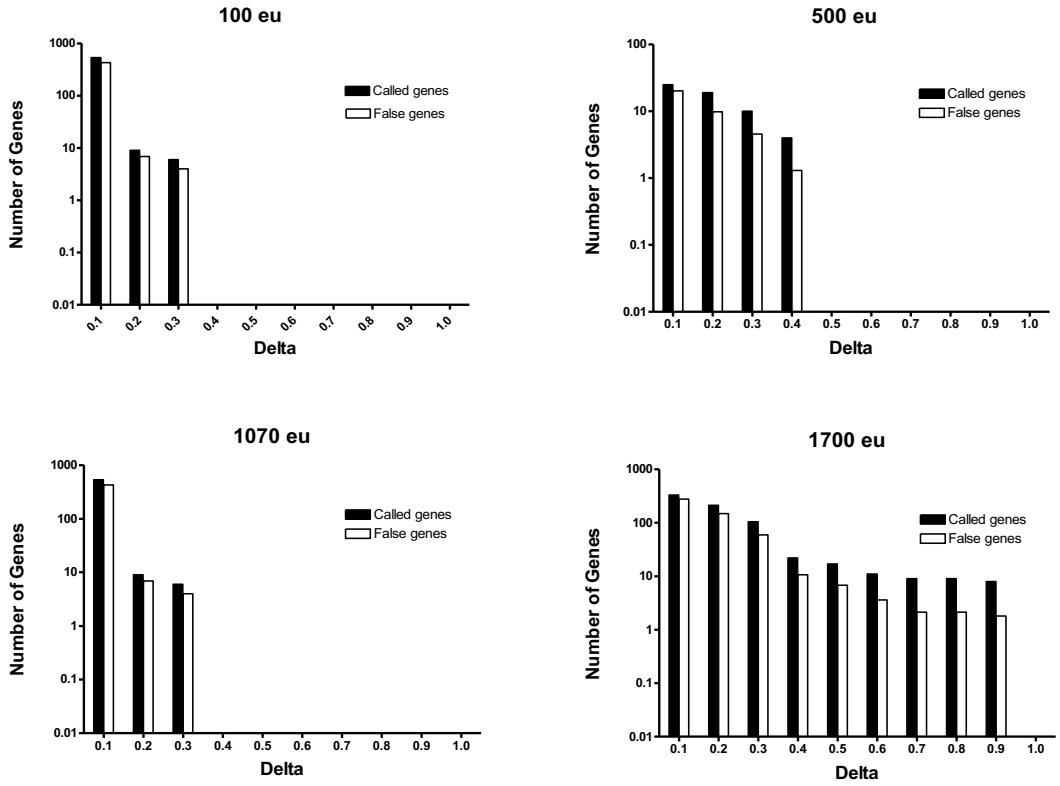

Figure 1: Graphical summery of Called and False genes in test groups
Four different levels of endotoxin contamination and at delta values from 0.1 to 1.0. The likelihood that expressed genes are falsely called greatly increases at low delta values.

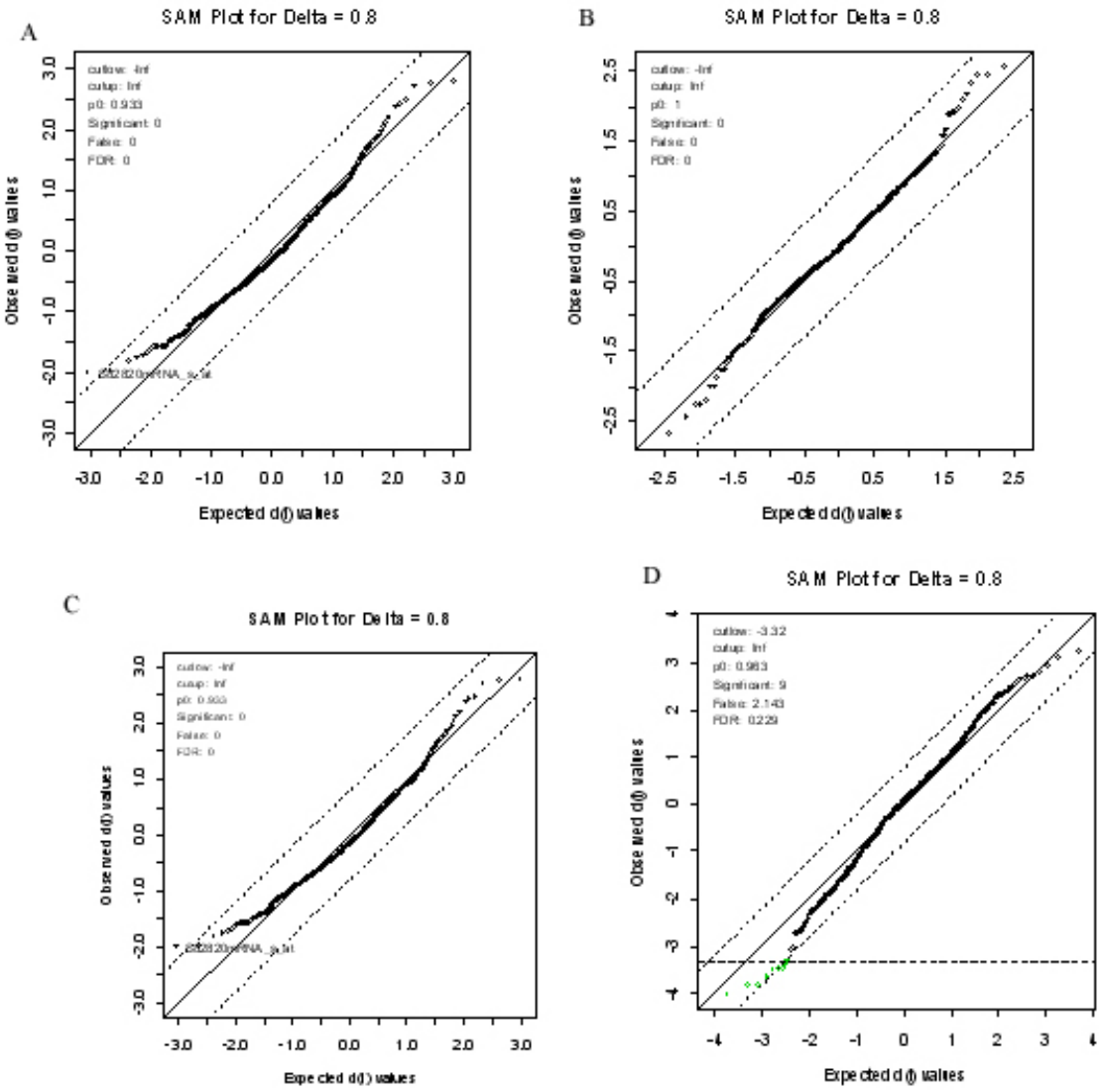

Figure 2: Significance Analysis of Microarrays (SAM) plots for Delta=0.8 for the four test groups.
A. 100 EU B. 500 EU. C. 1070 EU D. 1700 EU. The plots show observed verses expected gene expression based on microarray results using Delta=0.8. Green circles in panel D show genes that were underexpressed compared to the control group.

Table 1: Highlighted genes in green within 1700 EU Sam plot Delta=0.8 (Figure 2-D)

	Row	d.value	Std Dev.	Raw p	Q value	R fold
AFFX-BioB-5_at	92	-4.030499	0.07687599	0.001108494	0.4332442	0.7755654
AFFX-BioB-3_at	90	-3.808817	0.04049715	0.001690453	0.4332442	0.8630476
AFFX-BioB-M_at	94	-3.804259	0.09409570	0.001745878	0.4332442	0.7493999
rc_AI009132_at	614	-3.669686	0.06883686	0.002410974	0.4332442	0.8055146
U17697_s_at	801	-3.500990	0.09222072	0.002854372	0.4332442	0.7701263
AFFX-BioC-5_at	98	3.479195	0.03478881	0.002965221	0.4332442	0.8858284
X67654_at	976	-3.460842	0.13532880	0.003186920	0.4332442	0.6994577
M60322_at	435	-3.410747	0.02983115	0.003491756	0.4332442	0.8983299
S82820mRNA_s_at	763	-3.319908	0.30790052	0.004156852	0.4584595	0.4625030

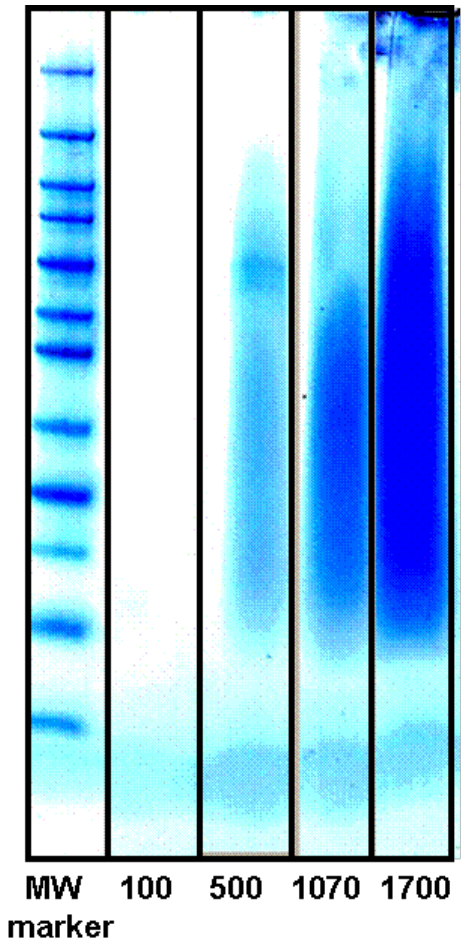

Figure 3: SDS PAGE of proteins contained in chitosan patches