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Gene Expression Analysis of the Bromobenzene Treated Liver with Non-hepatotoxic Doses in Mice

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Abstract

Bromobenzene (BB) is well known hepatotoxicant. Also, BB is an industrial solvent that arouses toxicity predominantly in the liver where it causes centrilobular necrosis. BB is subjected to Cytochrome P450 mediated epoxidation followed by either conjugation with glutathione, enzymatic hydrolysis or further oxidation. In this study, we focused on BB-induced gene expression at non-hepatotoxic dose. Mice were exposed to two levels of BB, sampled at 24 h, and hepatic gene expression levels were determined to evaluate dose dependent changes. When examining the toxic dose of BB treated group in other previous studies, genes related to heat shock protein, oxidative stress, and drug metabolism are expressed. Compared to these results, our study, in which non-toxic dose of BB was administrated, showed similar patterns as the toxic conditions above. The purpose of the study was to select genes that showed changes in relation to the differing dose through confirmation of the difference within transcriptomic boundaries, but those that are not detected by the existing classic toxicology tools in non-hepatotoxic dose.

Keywords: Toxicogenomics, non-hepatotoxic dose, gene expression, bromobenzene

Gene expression analyses in livers of experimental animals exposed to typical hepatotoxicants were studied in detail in numerous toxicogenomics research[1-6]. Benzene family is a widely studied chemical which induces hematotoxicity and myelotoxicity but is not a hepatotoxicants. Our laboratory identified characteristic changes at the gene expression and typical toxicological parameters (blood biochemistry, histopathology) induced with non-hepatotoxic doses of bromobenzene (BB). Recently, several studies reported that hepatotoxicity induce by bromobenzene[7-10]. Bioactivation of benzene in liver is capable to induce hepatomegaly. Benzene biotransformation is dependent on the dose, and the relative amount of muconic acid and other toxic metabolites decreases as the dose increases[11]. BB is an industrial solvent that arouses toxicity predominantly in the liver where it causes centrilobular necrosis. BB is subjected to Cytochrome P450 mediated epoxidation followed by either conjugation with glutathione, enzymatic hydrolysis or further oxidation. In this study, we focused on BB-induced gene expression at non-hepatotoxic dose. Mice were exposed to two dose levels of BB, sampled at 24 h, and hepatic gene expression levels were determined to evaluate dose dependent changes. We supposed that non-hepatotoxic doses have effect on early stage of hepatotoxicity.

Blood Biochemistry and Histopathology

H & E evaluation of stained liver sections revealed no significant histopathological finding in mice treated non-hepatotoxic doses of BB (Fig. 1). Levels of circulating enzymes were observed in mice receiving non-hepatotoxic doses of BB. Serum level of ALT and AST were not significantly changed (Fig. 2) following the BB treatments (Low 40 mg/kg or High 400 mg/kg). Twenty-four hours following a single intra-peritoneal injection of BB at 40 mg/kg (low dose) or 400 mg/kg (high dose), there was no hepatocellular generation, necrosis, inflammation and no evidence of blood biochemical change.

Gene Expression Analysis

DNA chip analysis was done to determine differences in hepatic gene expression between BB and vehicle-treated rats. For each of the approximately 7400 genes present on TwinChip Mouse 7.4 K (Digital Genomics, Korea). Gene expression profiles of interest were significantly up- or down-regulated in mice treated with BB when compared to control. Treat-

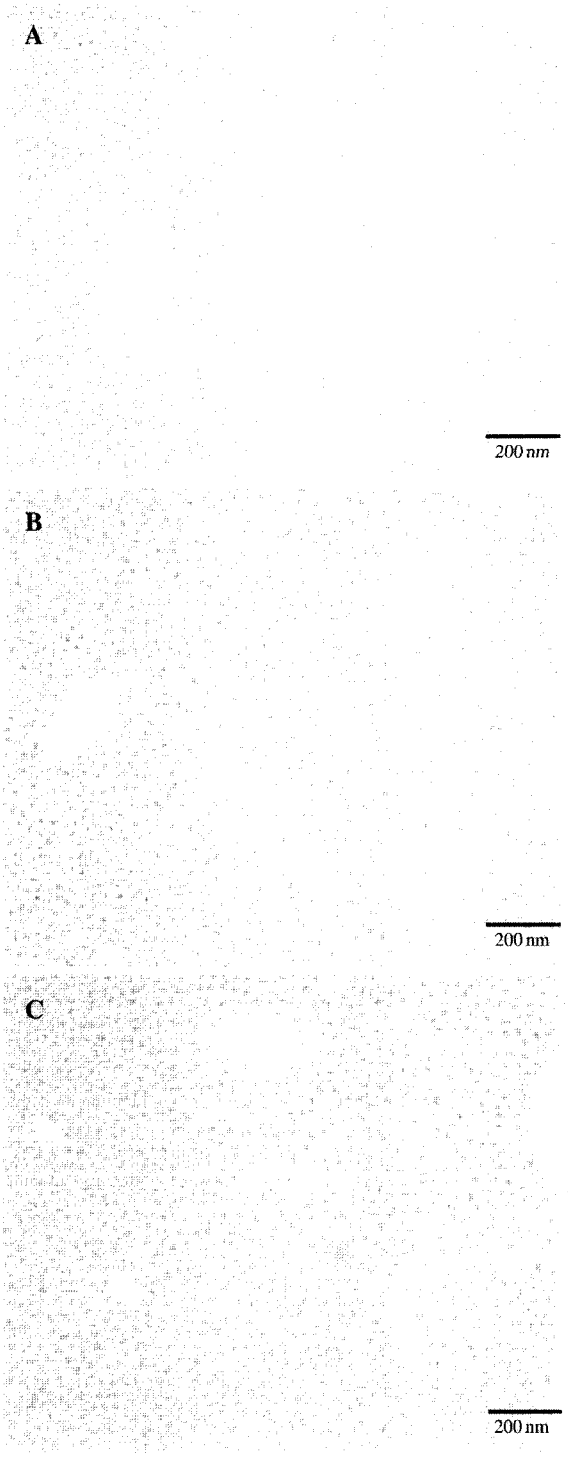

Fig. 1. Light photomicrographs of livers from a control male mouse and mice treated with Bromobenezene (A) control liver (B) Liver tissue of 40 mg/kg BB-treated mice, (C) Liver tissue of 400 mg/kg BB-treated mice. H & E 100×. There were no specific changes after injection regardless of low and high doses (non-hepatotoxic doses). We confirmed that these doses did not cause liver damage after 24 hrs.

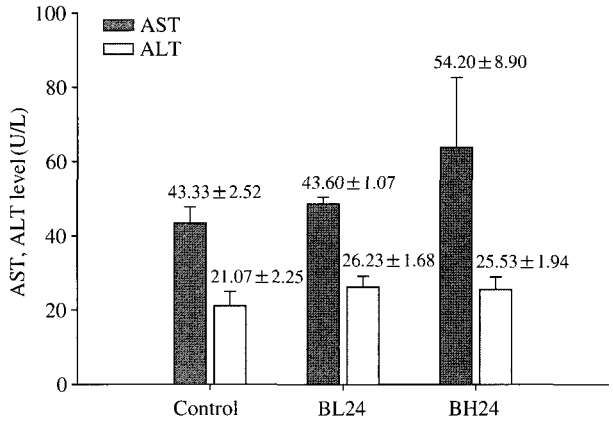

Fig. 2. Serum levels of AST and ALT enzymes, expressed as means and standard deviations of 3 animals/group. There were no specific changes after injection regardless of low and high doses (non-hepatotoxic doses). We also confirmed that these doses did not cause liver damage after 24 hrs in serum levels. AST : aspartate aminotransferase; ALT : alanine aminotransferase.

ment with non-hepatotoxic doses of BB caused the statistically significant, at least 2 fold up- or down regulation of many probe sets on the DNA chips. Hepatic gene expression was measured 24 h following BB treatment. As shown in Fig. 3, the number of expressed transcripts did vary between low and high dose group. ANOVA screening step was designed to identify significant ($p < 0.01$) treatment effects or treatment-by-dose interactions, thereby eliminating vehicle or other effects for which the only significant effect would be dose (Fig. 3C). Of the 158 that were considered to have a significant treatment effect or treatment-by-dose interaction. To represent the transcriptional response as a whole, a two-dimensional hierarchical clustering method was applied. A threshold of 2-fold change in gene expression was used as the cut-off value. Significantly increased or decreased gene's lists are showed at Table 1, 2.

Gene expression profiles showed that genes were 2 folds up- or down regulated in mice treated low dose of BB (Table 1); e.g. Cyp7b1, inhibition of DNA binding, hepatic nuclear factor 4, heat shock 70 kD protein, Cyp2a4, purine rich element binding protein B. In high dose treated group, gene expression profiles that genes were up- and down-regulated in mice treated high dose of BB (Table 2). We found commonly up- or down regulated genes in the low and high dose groups; e.g. Cyp4a14, Cyp2a4, sialyltransferase 9, selenium binding protein, Cyp4a10, BCL2, inhibitor of DNA binding protein B, purine rich element binding protein B, nudix-type motif 7, stearoyl-

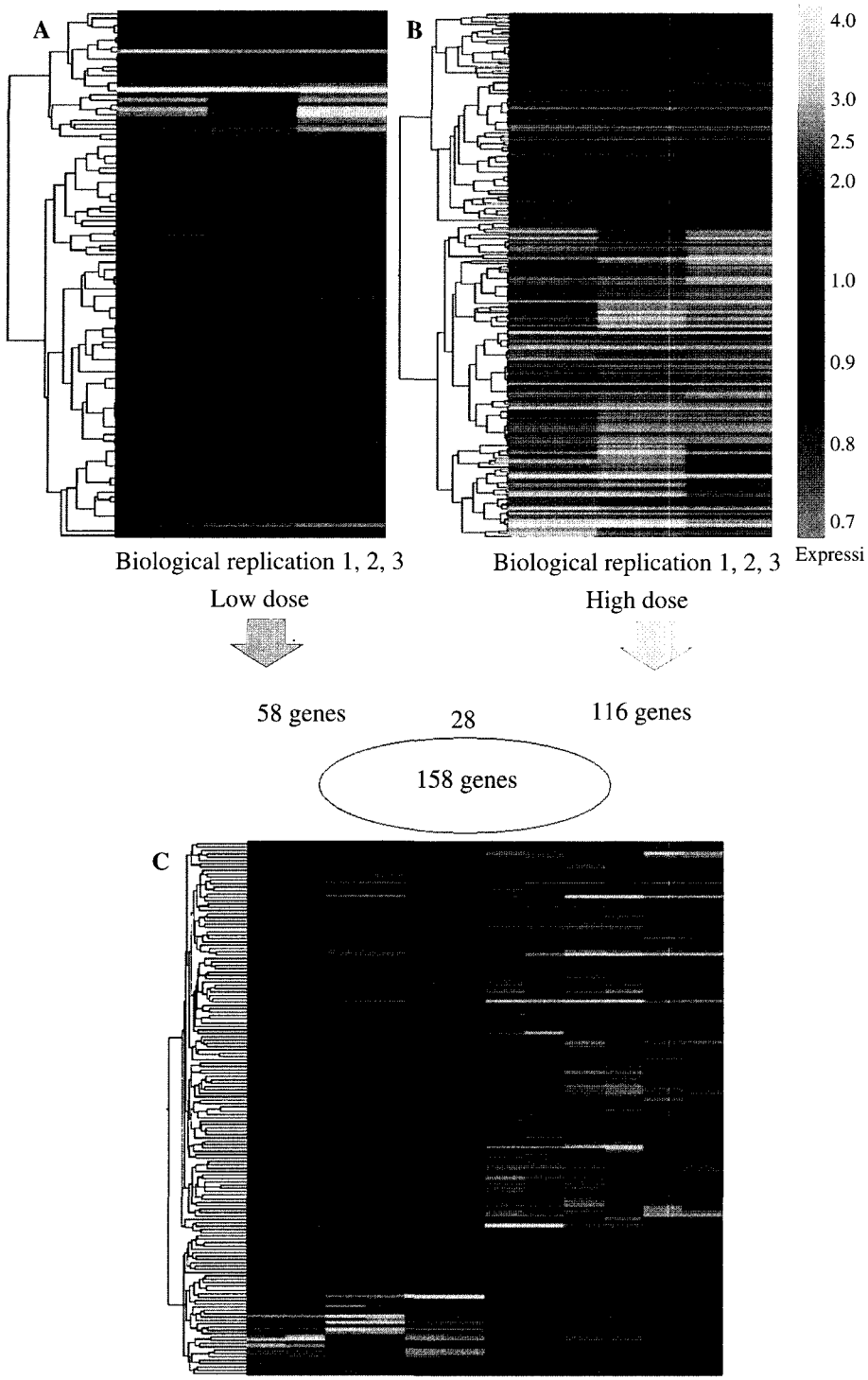

Fig. 3. Hierarchical cluster images showing the gene changes occurring in livers from mice treated with BB. 109 (A) and 234 (B) genes with p value of 0.01 were shown to be up or down-regulated by at least an individual (Mouse 7.4 K twin chips; Digitalgenomics, Korea). Total 158 genes with a p value of 0.01 were shown to be up- or down regulated by at least one dose condition.

coenzyme A desaturase 1 (Table 3). To obtain a molecular signature of the mechanism associated with mice exposed to BB, we used a hierarchical clustering analysis to group genes on the basis of expressed patterns (Fig. 4).

Discussion

The purpose of this study was to elucidate whether generation of hepatotoxicant associated gene expre-

Table 1. Significantly increased and decreased expression data

Expression ratio	Gene symbol	Description	GenBank ACC Number
2.109	Cyp7b1	Cyp7b1	AW261589
1.888	Idb2	Inhibitor of DNA binding 2	AI848408
1.852	BC013667	CDNA sequence BC013667	AI047920
1.823	Cyp7b1	Cyp7b1	AI156457
1.743	Idb3	Inhibitor of DNA binding 3	AI839283
1.720	Hnf4a	Hepatic nuclear factor 4, alpha	AI893936
1.684	Hspa5	Heat shock 70kD protein 5	AI507545
0.568	Cyp2a4	Cyp2a4	AI893559
0.543	Purb	Purine rich element binding protein B	AI503584
0.524	Tesk1	Testis specific protein kinase 1	AI449716

10 genes with p value of 0.01 were shown to be regulated ±2-fold or more by at least an individual of Low dose treatment.

Table 2. Significantly increased and decreased expression data

Expression ratio	Gene symbol	Description	GenBank ACC Number
4.391	Idb2	Inhibitor of DNA binding 2	AI848408
3.510	Nudt7	Nudix-type motif 7	AI527308
3.222	Fga	Fibrinogen, alpha polypeptide	W89831
3.065	Apcs	Serum amyloid P-component	AI527294
2.739	Idb2	Inhibitor of DNA binding 2	AI322367
2.719	Armet	Arginine-rich, mutated in early stage tumors	AI838290
2.409	Scd1	Stearoyl-Coenzyme A desaturase 1	AI853169
2.294	Hspa5	Heat shock 70 kD protein 5	AI507545
2.201	Fgb	Fibrinogen, B beta polypeptide	AI327250
2.146	Srebf1	Sterol regulatory element binding factor 1	AI482261
2.064	AI303526	Similar to Ac1873	AI303526
0.464	Fmo1	Flavin containing monooxygenase 1	AW319979
0.456	Gstm2	Glutathione S-transferase, mu 2	AI838612
0.445	Gpt1	Glutamic pyruvic transaminase 1, soluble	AI427571
0.430	Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 1	AI956627
0.415	Fmo2	Flavin containing monooxygenase 2	AW261476
0.401	Selenbp1	Selenium binding protein 1	AI322955
0.394	Slco1a4	Solute carrier organic anion transporter family	AI843160
0.386	Ppp1r3c	Protein phosphatase 1, regulatory (inhibitor)	AI649217
0.312	Cyp4a10	Cyp4a10	AW318974
0.298	Siat9	Sialyltransferase 9	AI841090
0.202	Lpin1	Lipin 1	AI846934
0.147	Cyp4a14	Cyp4a14	AI893426
0.144	Cyp2a4	Cyp2a4	AI893559

24 genes with p value of 0.01 were shown to be regulated ±2-fold or more by at least an individual of High dose treatment

ssion with phenotype independent doses, using DNA chip technology, would get information of early step of hepatotoxicity. We believe our strategy for the treatment of phenotype-independent dose has advantages in early response against hepatotoxicants. Toxicity of BB depends on bioactivation, primarily by Cyp2e1 in the liver[11,12]. Benzene biotransformation is dependent on the dose, and the relative amount of muconic acid and other toxic metabolites decreases as the dose increases[13]. However, in this study we could not detect significant Cyp2e1 induction with BB treatment.

At the low dose (40 mg/kg) of BB the expression levels of 9 genes were significantly changed (Table 1). Genes involved in heat shock, nuclear factor and drug metabolizing enzyme. At high dose (400 mg/kg) BB 24 genes were significantly changed and included genes involved heat shock protein, stress related protein response were induced, whereas several drug metabolizing enzymes (e.g. Cytochrome P450s, FMO) were repressed. Especially, different CYP (4a10, 4a14, 2a4) were down regulated at high dose of BB treatment. When examining the toxic dose of BB treated group in other previous studies, genes related to heat shock protein, oxidative stress, and drug metabolism are expressed[3,6,14,15]. Compared to these

Table 3. Significantly increased and decreased expression data

Expression ratio		Gene symbol	Description	GenBank ACC Number
Low dose	High dose			
2.361	0.509	Cyp4a14	Cytochrome P450, family 4, subfamily a, polypeptide 14	AI893426
1.928	0.506	Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 5	AI893559
1.946	0.526	Siat9	Sialyltransferase 9	AI841090
1.821	0.561	Selenbp1	Selenium binding protein 1	AI322955
1.716	0.690	Cyp4a10	Cytochrome P450, family 4, subfamily a, polypeptide 10	AW318974
1.589	0.723	Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 1	AI956627
0.599	1.412	Idb2	Inhibitor of DNA binding 2	AI848408
0.621	1.561	Purb	Purine rich element binding protein B	AI503584
0.571	1.449	Nudt7	Nudix (nucleoside diphosphate linked moiety X)-type motif 7	AI527308
0.520	1.470	Scd1	Stearoyl-Coenzyme A desaturase 1	AI853169

2-fold up and down genes from low and high dose treatment filtered from ANOVA ($p < 0.01$). Values shown are the average of expression ratio. Both values are shown when a gene was induced at one condition, and repressed at the other.

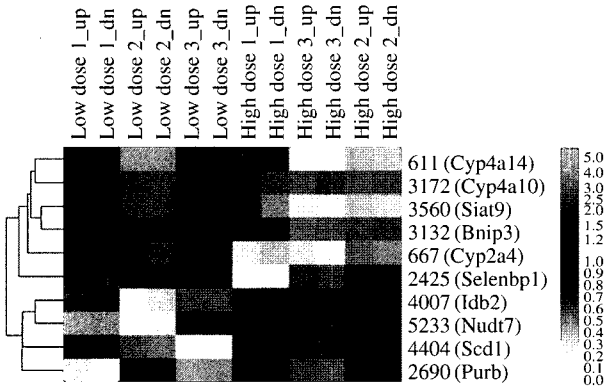

Fig. 4. Hierarchical clustering view of individual group and genes from Table 3. Bar graph revealed from expression ratio.

results, our study, in which non-toxic dose of BB was administrated, showed similar patterns as the toxic conditions above. The focus of the study was to select genes that showed changes in relation to the differing dose through confirmation of the difference within transcriptomic boundaries, but those that are not detected by the existing classic tools (e.g. blood biochemistry, histopathology etc.) in non-hepatotoxic dose. Throughout the comparisons of low and high dose groups, 10 genes that showed at least two-fold increase or decrease were seen. Although phenotypes were not shown in this study, as Figure 4 shows, genes show very different aspect of gene expression in the two dose groups. Thus, we propose these 10 genes to provide useful information for toxicity prediction through dose distribution. Expressed genes, by showing the extreme opposite changes of expression in condition of dose differing by 10 fold, are prospected to be biomarkers at a level of sensitivity that is difficult to recognize by existing method. Despite the fact that the reasons for changes for all the mechanisms are limited in its explanation, the genes being expressed in opposite patterns were an interesting result to observe that in controlling the expression of genes *in vivo*, it has gone over the threshold. Further study is needed, but this study fully showed the concept of predictive toxicology by BB administration. Through such molecular signature, the recognition of gene expression at genomic level is suggested to be extremely sensitive.

Methods

Animals

Approximately 10-week-old C57BL/6J male mice (Orient, Korea) were kept in a 12-h light/dark cycle animal room with controlled temperature and humidity for 2 week prior to experiment. The mice maintained on a standard diet and weighed from 24 to 27g (mean $\pm$ SD; 25.75 ± 0.97) in these experiments. Thus the terminal body weights and liver weights were recorded. Corn oil- (vehicle) and BB-treated groups consisted of 3 mice at each point, respectively. Those were exposed intraperitoneally to two levels (40 or 400 mg/kg) of BB that have previously been shown to be non-hepatotoxic and sampled at 24 h.

Chemicals

BB and corn oil were purchased from Sigma-Aldrich. BB were suspended or dissolved in corn oil. Trizol was purchased from Invitrogen, Life Technologies

Biochemical Analysis

After the mice were anesthetized with diethyl ether, blood was gathered through the inferior vena cava. For biochemical parameters, commercial kits (kits58-

UV, kits 59-UV; Sigma, U.S.A.) were used in this study. The activities of alanine aminotransferase (AST), aspartate aminotransferase (ALT) were measured using the Shimadzu automatic biochemistry analyzer (Shimadzu, Japan) at each time points as indicated above.

Histopathology

The liver samples were harvested from the 10-12 weeks old mice, fixed in 10% neutral buffered formalin, and embedded in paraffin. For histopathological analysis, the sections were cut 4 μm in thickness (RM2165 Microtome, Leica, Germany), stained with hematoxylin and eosin (H & E), and examined with a light microscope (Nikon E400, Japan).

Isolation of RNA

The left lateral lobe of the liver was processed for RNA extraction. For cDNA microarray analysis, total RNA was extracted using TRIzol reagent and purified using RNeasy total RNA isolation kit (Qiagen, U.S.A.) according to the manufacturer's instructions. Total RNA was quantified by NanoDrop (NanoDrop, U.S.A.) and its integrity was assessed by running 2100 Bioanalyzer (Agilent, Germany). The remaining portion of the liver was retained frozen for future study.

cDNA Microarray

Fluorescent-labeled cDNA for microarray analysis was prepared by the reverse-transcription of total RNA in the presence of aminoallyl-dUTP followed by the coupling of Cy3 or Cy5 dyes (Amersham Pharmacia, Sweden). Single-stranded cDNA probes were purified using a PCR purification kit (Qiagen, U.S.A.). Probes were resuspended in hybridization solution containing 50% formamide, 5X SSC, 0.1% SDS. The TwinChip Mouse-7.4 K cDNA microarray (Digital Genomics, Korea) was hybridized with the fluorescent-labeled cDNAs at 42°C in a humid chamber.

Analysis of Fluorescence Spots

After the washing procedure the slides were scanned using ScanArray Lite (PerkinElmer Life Sciences, U.S.A.). Scanned images were analyzed with GeneSpring Software version 7.0 (Silicon Genetics, U.S.A.) to obtain gene expression ratios. Logged gene expression ratios were normalized by LOWESS regression. Only genes whose expression was above 2-fold induction or below 2-fold repression by BB treatment compared with controls were considered for statistical analysis. Significant differences between control and treatment doses were determined using t-test. P values <0.05 were considered statistically significant.

Cluster Analysis, Data Annotation

Lists of the significantly differentially regulated genes upon BB treatment were further analyzed using Genespring software. Hierarchical clustering was applied to identify similarly regulated groups and to determine treatment-related effects. The hierarchical clustering of Genespring program class together experiments of genes by clustering based upon expression profile similarities. Information was collected from different databases, including Unigene from the National Center for Biotechnology Information (NCBI) or NetAffx Analysis Center (Liu *et al.*, 2003).

Statistical Analysis

Data are expressed as the mean $\pm$ SD of samples. Comparisons of differences were performed by using analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Acknowledgements

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