Table 5. List of genes that showed expression of more than 3-fold of control by APAP at 24 hr at 12-week-old rats.

Probe Set ID	Gene Symbol	Gene ID	Fold change 6W	Fold change 12W	Gene Title
1368247_at	Hspa1a /// Hspa1b_mapped	NM_031971		37.3	heat shock 70kD protein 1A /// heat shock 70kD protein 1B (mapped)
1370912_at	Hspa1b_mapped	BI278231		30.0	heat shock 70kD protein 1B (mapped)
1368321_at	Egr1	NM_012551		17.3	early growth response 1
1372510_at	Srxn1	AI172302	2.7	17.0	Sulfiredoxin 1 homolog (S. cerevisiae)
1383302_at	Dnajb1_predicted	BM384926		12.4	DnaJ (Hsp40) homolog, subfamily B, member 1 (predicted)
1387060_at	Klf6	NM_031642		10.1	Kruppel-like factor 6
1370080_at	Hmox1	NM_012580		9.3	heme oxygenase (decycling) 1
1368160_at	Igfbp1	NM_013144	4.0	8.8	insulin-like growth factor binding protein 1
1370436_at	LOC246263	AF062389	3.1	8.4	kidney-specific protein (KS)
1386321_s_at	Trib3	H31287	3.9	7.9	tribbles homolog 3 (Drosophila)
1388986_at	---	AI598339		7.8	Transcribed locus
1367856_at	G6pdx	NM_017006	2.9	7.8	glucose-6-phosphate dehydrogenase X-linked
1389528_s_at	Jun	BI288619		7.7	Jun oncogene
1367795_at	Ifrd1	NM_019242		7.4	interferon-related developmental regulator 1
1388271_at	---	BM383531	4.4	7.3	---
1374404_at	Jun	BI288619		6.9	Jun oncogene
1388722_at	Dnajb1_predicted	AA945704		6.8	DnaJ (Hsp40) homolog, subfamily B, member 1 (predicted)
1368308_at	Myc	NM_012603		6.4	myelocytomatosis viral oncogene homolog (avian)
1368947_at	Gadd45a	NM_024127		6.4	growth arrest and DNA-damage-inducible 45 alpha
1386958_at	Txnrd1	U63923		6.4	thioredoxin reductase 1
1368121_at	Akr7a3	NM_013215	5.2	6.3	aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)
1368718_at	Aldh1a4	M23995		6.2	aldehyde dehydrogenase family 1, subfamily A4
1370688_at	Gclc	J05181	2.7	5.9	glutamate-cysteine ligase, catalytic subunit
1375852_at	Hmgcr	BM390399		5.9	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
1371785_at	Tnfrsf12a	BI303379		5.3	tumor necrosis factor receptor superfamily, member 12a
1368124_at	Dusp5	NM_133578		5.3	dual specificity phosphatase 5
1398343_at	Dnaja4	AI104324		5.0	DnaJ (Hsp40) homolog, subfamily A, member 4
1374433_at	RGD1563902_predicted	BI301532		4.9	Similar to Ferritin light chain (Ferritin L subunit) (predicted)
1369590_a_at	Ddit3	NM_024134		4.7	DNA-damage inducible transcript 3
1398791_at	Txnrd1	NM_031614		4.6	thioredoxin reductase 1
1387282_at	Hspb8	NM_053612		4.5	heat shock 22kDa protein 8
1387599_a_at	Nqo1	J02679	4.2	4.5	NAD(P)H dehydrogenase, quinone 1
1387022_at	Aldh1a1	NM_022407	2.2	4.5	aldehyde dehydrogenase family 1, member A1
1390026_at	Bag3	AI231792		4.3	Bcl2-associated athanogene 3
1370030_at	Gclm	NM_017305	4.4	4.3	glutamate cysteine ligase, modifier subunit
1388850_at	Hspca	BG671521		4.3	heat shock protein 1, alpha
1388898_at	Hsph1	AI236601		4.2	heat shock 105kDa/110kDa protein 1
1384427_at	Mdm2_predicted	BI296301		4.2	transformed mouse 3T3 cell double minute 2 homolog (mouse) (predicted)

Table 5. Continued.

Probe Set ID	Gene Symbol	Gene ID	Fold change 6W	Fold change 12W	Gene Title
1370583_s_at	Abcb1 /// Abcb1a	AY082609	3.6	4.1	ATP-binding cassette, sub-family B (MDR/TAP), member 1 /// ATP-binding cassette, sub-family B (MDR/TAP), member 1A
1368147_at	Dusp1	BE110108		4.1	dual specificity phosphatase 1
1388721_at	Hspb8	BG380282		4.1	heat shock 22kDa protein 8
1368213_at	Por	AI407454		4.1	P450 (cytochrome) oxidoreductase
1372523_at	Gclc	AA892770	2.2	4.0	glutamate-cysteine ligase, catalytic subunit
1386995_at	Btg2	BI288701		4.0	B-cell translocation gene 2, anti-proliferative
1386922_at	Ca2	AI408948	4.5	4.0	carbonic anhydrase 2
1387283_at	Mx2	NM_134350	2.0	3.9	myxovirus (influenza virus) resistance 2
1372389_at	Ier2	BF420059		3.6	immediate early response 2
1371237_a_at	Mt1a	AF411318	4.5	3.6	metallothionein 1a
1372261_at	---	AI409067	2.7	3.5	Transcribed locus
1373473_a_at	Napl1l	BM386384	2.1	3.5	nucleosome assembly protein 1-like 1
1367733_at	Ca2	NM_019291	3.2	3.5	carbonic anhydrase 2
1373810_at	Pla2g12a_predicted	BF284175	3.6	3.5	phospholipase A2, group XIIA (predicted)
1377092_at	---	BF389682		3.4	Transcribed locus
1388622_at	Nol5a	AW535890		3.4	nucleolar protein 5A
1389573_at	Chac1_predicted	AI170665		3.4	ChaC, cation transport regulator-like 1 (E. coli) (predicted)
1375895_at	---	BI275908		3.3	CDNA clone IMAGE:7302535, with apparent retained intron
1368332_at	Gbp2	NM_133624		3.3	guanylate nucleotide binding protein 2
1369061_at	Gsr	NM_053906	2.2	3.3	glutathione reductase

Fold changes in 6-week-old rats are also shown for values higher than 2.

which are Cyp3A inducers, increased APAP hepatotoxicity (Jaw and Jeffery, 1993; Madhu *et al.*, 1993; Jue *et al.*, 2002; Guo *et al.*, 2004). Therefore, it would be reasonable to assume that the difference in basal expression levels of Cyp3A13 between young and adult rats accounts for the difference in susceptibility to APAP toxicity. Cyp2E1 is another enzyme responsible for biotransformation, i.e., inhibition of Cyp2E1 decreased the production of a reactive metabolite in the liver microsome from humans (Hazai *et al.*, 2002), although this enzyme did not show an age-difference. Based on the present results, participation of CYP3A is considered to be larger than that of CYP2E in the rat.

GSTYc2 was one isoform of glutathione *S*-transferase, and was known to play an important role in aflatoxin detoxification (Buetler and Eaton, 1992; Hayes *et al.*, 1992 and 1994). Although the role of GSTYc2 in APAP detoxification has not been investigated, it could have some role as GST conjugation, a key detoxification pathway of the reactive intermediate of APAP.

When the total of the GST and SH-contents in the liver were measured, they were found to be higher in adult than in young under the control condition, while their reduction by APAP-treatment was larger in adult than in young. This could be explained by our assumption from gene expression analysis that the production of active metabolite is higher and the rate of the supply of the detoxification enzyme is lower in adult rats.

The age-related difference in the number of mobilized genes at 24 hr after APAP-treatment was considered to be correlated with the phenotype. Namely, most of these changes in the expression reflected the results of pathological changes. When the precise analysis of the genes showing age-related difference was made in terms of time- and dose-related responses, another view emerged. Among the genes with age-related difference at 24 hr after APAP-treatment, there were not only genes with differing levels of expression but also differences in the response time.

Table 6. List of genes that showed expression of less than 0.3-fold of control by APAP at 24 hr in 12-week-old rats.

Probe Set ID	Gene Symbol	Gene ID	Fold change 6W	Fold change 12W	Gene Title
1371412_a_at	Nrep	BE107450	0.25	0.06	Neuronal regeneration related protein
1386977_at	Ca3	NM_019292	0.20	0.08	carbonic anhydrase 3
1374251_at	---	AA893192		0.11	Transcribed locus
1367896_at	Ca3	AB030829		0.13	carbonic anhydrase 3
1369864_a_at	Sds	NM_053962	0.47	0.17	serine dehydratase
1387665_at	Bhmt	U96133	0.40	0.18	betaine-homocysteine methyltransferase
1374244_at	LOC501038	AI411141	0.35	0.18	Ab2-060
1376976_at	Sectm1	AI009823		0.18	secreted and transmembrane 1
1373686_at	Serpina6_mapped	AA893495	0.42	0.19	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6 (mapped)
1389681_at	Pvrl2	BI296388	0.24	0.21	Poliovirus receptor-related 2 (herpesvirus entry mediator B)
1368171_at	Lox	NM_017061	0.33	0.21	lysyl oxidase
1374531_at	---	AA926305		0.22	Transcribed locus
1368826_at	Comt	NM_012531		0.22	catechol-O-methyltransferase
1393221_at	RGD1564865_predicted	AA866264		0.23	similar to 20-alpha-hydroxysteroid dehydrogenase (predicted)
1368458_at	Cyp7a1	NM_012942	0.45	0.23	cytochrome P450, family 7, subfamily a, polypeptide 1
1368172_a_at	Lox	BI304009		0.25	lysyl oxidase
1373778_at	---	BE349670	0.32	0.26	Transcribed locus
1368270_at	Apobec1	NM_012907		0.26	apolipoprotein B editing complex 1
1373814_at	RGD1310066	BI291270		0.26	similar to mKIAA1002 protein
1398759_at	Tgfb1i4	NM_013043	0.34	0.27	transforming growth factor beta 1 induced transcript 4
1388583_at	Cxcl12	BF283398	0.42	0.27	chemokine (C-X-C motif) ligand 12
1368778_at	Slc6a6	NM_017206	0.34	0.27	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
1368543_at	Nox4	NM_053524		0.27	NADPH oxidase 4
1375560_at	RGD1310475_predicted	AA945579		0.28	similar to RIKEN cDNA 0610010D20 (predicted)
1388426_at	Srebf1	BF398848	0.50	0.28	sterol regulatory element binding factor 1
1387053_at	Fmo1	NM_012792		0.28	flavin containing monooxygenase 1
1375216_at	Pvrl2	AA850909	0.31	0.28	poliovirus receptor-related 2 (herpesvirus entry mediator B)
1387263_at	Pklr	NM_012624		0.28	pyruvate kinase, liver and red blood cell
1374493_at	---	H35017		0.29	Transcribed locus
1369664_at	Avpr1a	NM_053019	0.36	0.30	arginine vasopressin receptor 1A
1377015_at	Me3_predicted	BF395080	0.43	0.30	Malic enzyme 3, NADP(+)-dependent, mitochondrial (predicted)
1371137_at	Acox2	X95189		0.30	acyl-Coenzyme A oxidase 2, branched chain
1372684_at	Smtn	AW435036		0.30	smoothelin
1387123_at	Cyp17a1	NM_012753	0.30	0.30	cytochrome P450, family 17, subfamily a, polypeptide 1
1367729_at	Oat	NM_022521	0.30	0.30	ornithine aminotransferase
1374625_at	Hes6	AI176616	0.47	0.30	hairy and enhancer of split 6 (Drosophila)
1373975_at	LOC368066	AI232716		0.30	similar to thioether *S*-methyltransferase

Fold changes in 6-week-old rats are also shown for values lower than 0.5.

Especially, genes related to defensive responses to stress, e.g., heat shock protein, Hmox-1, Gadd45, and thioredoxin reductase were markedly induced by APAP in young rats at 6 or 9 hr after treatment, whereas adult rats did not respond as yet. This is considered to be due to the age-related difference in the toxicokinetics, i.e., young rats responded to APAP in the early stage of exposure because of the low SH level but recovered at 24 hr because of the high detoxification capacity.

In the case of CCl4, its active metabolite is reported to be produced by CYP2E1 (Parkinson, 2001) and this fits the present observation that neither the expression of CYP2E1 nor hepatotoxicity of CCl4 showed a difference between the ages. Although GSH conjugation should play an important role in their detoxification, the participation of GSTYc2 is considered to be less important for CCl4 in rats. It has been reported that both *N*-acetylation and CYP2E1 are involved in hepatotoxicity of INH (Parkinson, 2001). In the present study, no age-related difference was found in the expression of *N*-acetylating enzymes and CYP2E1, in accordance with the same extent of hepatotoxicity of INH for both ages. However, one should be careful in these conclusions, since phenotypical changes by INH and CCl4 were minimal in the present

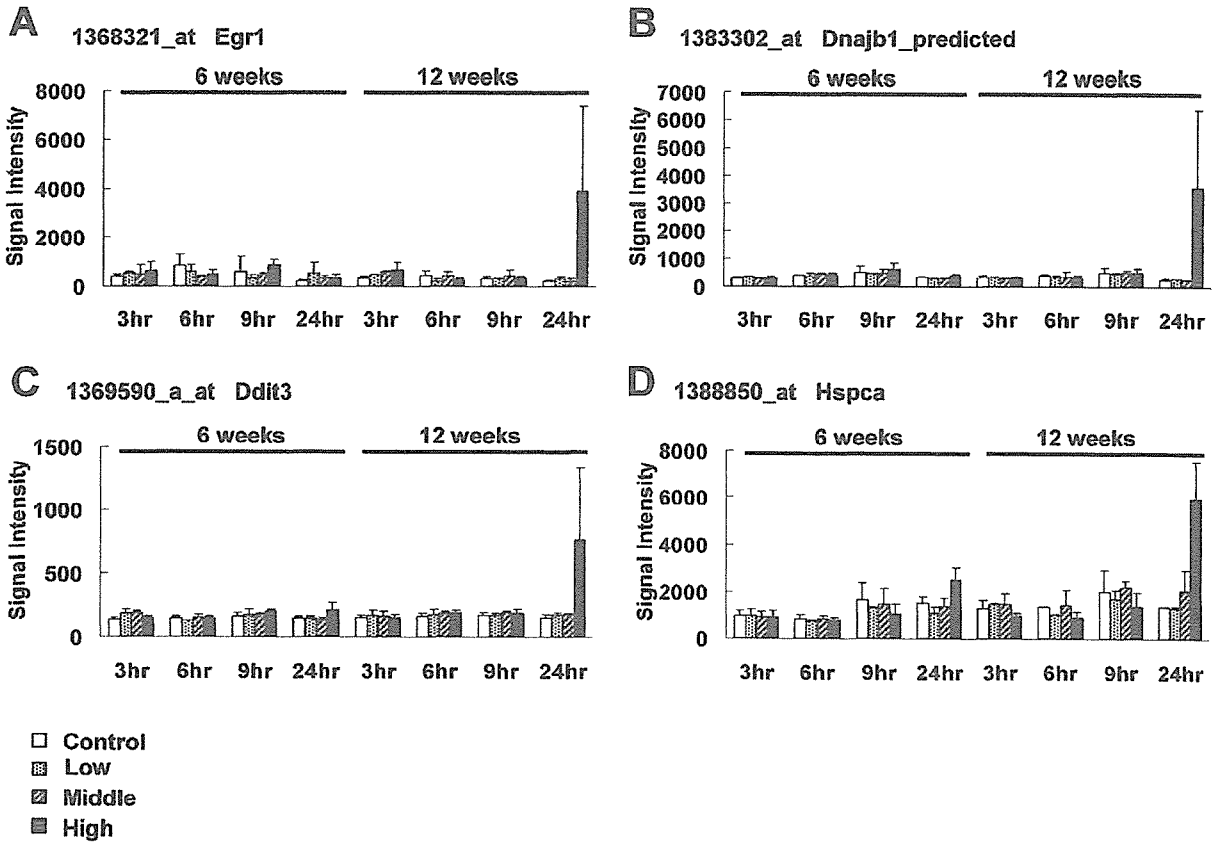

Fig. 3. Time- and dose-dependent effects of APAP on expression of Egr1 (A), Dnajb1 (predicted) (B), Ddit3 (C), and Hspca (D). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg of APAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip® RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and then expressed as mean ± SD (N=3). Note that the expression of these genes was markedly increased by APAP-treatment at 24 hr in adult (12-week-old) rats.

condition, which was optimized for their repeated administration for up to 28 days in our standard protocol.

The TGP database has huge gene expression data as well as the traditional toxicity study parameters, currently for over 100 chemicals. Although the main purpose of the project is to predict toxicity in the early stage of drug development, it is also useful for drawing some mechanistic insight in observed toxicity. In the present study, it would have been impossible to pick up such genes responding in a different time course with age, unless a multi-time, multi-dose protocol was employed. In general, dose or time points tend to be limited in the collection of toxicological data by microarray due to the cost. The present study has clearly showed the advantage of our protocol with enough numbers of data points. This promises a good use for our database now being created in the project.

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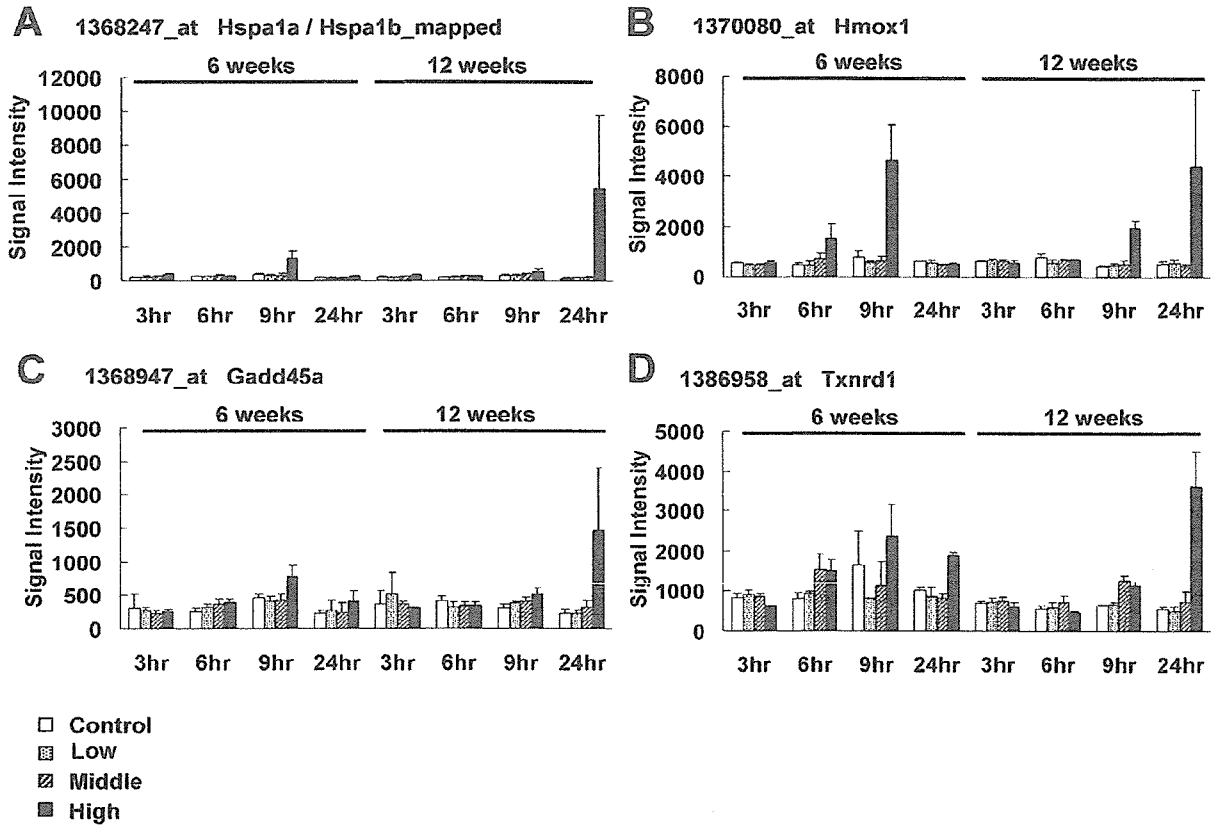

Fig. 4. Time- and dose-dependent effects of APAP on expression of Hsp a1a/a1b (A), Hmox-1 (B), Gadd45 (C), and Txnrd (D). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg of APAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip® RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and then expressed as mean ± SD (N=3). Note that the induction of these genes by APAP at 24 hr was larger in adult (12-week-old) than young (6-week-old) rats, whereas the young rat responded to APAP earlier.

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EVALUATION OF METHODS FOR DURATION OF PRESERVATION OF RNA QUALITY IN RAT LIVER USED FOR TRANSCRIPTOME ANALYSIS

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ABSTRACT — In The Toxicogenomics Project, about 150 chemicals are administered to rats, and gene expression in the liver analyzed by Affymetrix GeneChip and stored in the database. As the quality of RNA greatly influences the accuracy of gene expression data, conditions of the storage of the sample are very important. Recently, an RNA stabilization solution, RNAlater®, has become commercially available. In this study, the new storage method was compared with the traditional storage method (stored in freezer or liquid nitrogen) under various conditions by looking at the degradation of RNA assessed by its total yield, OD260/280 ratio, 28S/18S ratio, and quantity of β-actin. It was confirmed that RNAlater® preserved the liver tissue sample by maintaining the quality of RNA for one year (in liquid N_2 or −80°C), for 3 days (4°C), or for 2 hr (room temperature) without degradation of RNA. Quality of RNA samples dissolved in buffer RLT and stored at −20°C tended to decrease, but samples stored at −80°C were almost equivalent to those stored in liquid nitrogen. In conclusion, we recommend the following procedure for preservation of liver tissue for extraction of RNA: 1) tissues removed should be put into chilled RNAlater® as soon as possible; 2) samples in RNAlater® must be stored overnight or longer at 4°C and can be left for as long as 2 weeks without freezing; 3) samples in RNAlater® can be stored for at least one year under less than −20°C and 4) samples dissolved in buffer RLT can be preserved at least for one year under −80°C.

KEY WORDS: mRNA stability, Rat, Liver

INTRODUCTION

The Toxicogenomics Project is a 5-year collaborative project conducted by the National Institute of Health Sciences (NIHS) and 17 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). In April 2005, some rearrangements were made and the project is now conducted by NIHS, the National Institute of Biomedical Innovation, and 15 pharmaceutical companies. Its aim is to construct a large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medical compounds, have been selected, and administered to rats to obtain gene expression profile in the liver (also in kidney in some cases) using Affymetrix GeneChip in addition to traditional toxicological measures, including body/organ weight, blood biochemistry, hematology and histopathology. In order to overcome the species difference, bridging experiments are also performed using primary cultures of human and rat hepatocytes. Needless to say, the quality of the data should be good in order to create a drug safety predicting system with high accuracy. Since RNA is quite labile, it is quite important to preserve tissues, stabilizing the RNA for experiments of gene expression profiling. So far, isolated tissues had been promptly frozen in liquid nitrogen and then kept as such or in a deep

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freezer. However, this is costly and may be risky if the freezer is in trouble, and prompt melting is required to ensure the quality of RNA. Recently, a new RNA stabilization solution, RNAlater® (Ambion, Austin, TX, USA) has become commercially available and is used in many laboratories (Grotzer *et al.*, 2000; Wang *et al.*, 2001; Rodrigo *et al.*, 2002; Mutter *et al.*, 2004). According to the manufacturer's notes, RNAlater® makes it possible to preserve tissues at a relatively high temperature and to repeat freezing-thawing, keeping the RNA stabilized. This is quite convenient, especially in our project, where vast numbers of samples are processed. To assess the usefulness of this product, we checked the following points:

1) Effects of the conditions (temperature and period) of fixing before freezing the sample.
2) Long-term preservation of samples fixed with RNAlater® at 4°C overnight compared with traditional procedures (liquid N_2 or freezers).
3) Effects of freezing-thawing on the RNA quality in the sample.
4) Stability of RNA in the sample dissolved in Buffer RLT.

The quality of RNA was estimated by the amount of total RNA, the OD260/280 ratio, 28S/18S ratio on agarose electrophoresis, and the amount β-actin mRNA quantified by RT-PCR. Based on the results of these assessments, we determined the standard protocol of sample preservation for our project.

MATERIALS AND METHODS

Preparation of total RNA

Male Sprague-Dawley rats were purchased from Charles River Japan Inc., (Atsugi, Japan) and used at 10 weeks of age. The animals were killed by exsanguination under anesthesia, and the livers removed quickly and provided for each experiment. The weights of microtubes with 0.5 mL RNAlater® were measured in advance. A piece of liver tissue (~50 mg) was cut out by using a disposable biopsy punch (ϕ=5 mm, Kai Industries, Gifu, Japan) and put into the pre-weighed microtube with or without RNAlater®. Total RNA was isolated using the RNeasy kit. The liver tissue was homogenized in 0.5 mL RLT with a Mill Mixer (Qiagen) and zirconium beads. The lysate corresponding to ca. 20 mg tissue was transferred to a microtube with an equivalent volume of chloroform, and 3 volumes of TRIzol LS Reagent (Invitrogen life technologies, Carlsbad, CA, USA) were added to the homogenate. The mixture was then shaken vigorously for 30 s, let stand at room temperature for 5 min and centrifuged thereafter at 12,000 *g* for 15 min. The resultant aqueous layer was transferred to a new tube. To the aqueous solution, an equivalent volume of 50% ethanol was added and mixed by pipetting. The whole mixture was transferred to an RNeasy mini column. The following procedure was performed according to the user's manual. Finally, absorbance of the purified RNA fraction was measured spectrophotometorically at 260 and 280 nm.

Electrophoresis of total RNA

To confirm the quality of total RNA, the total RNA solution (1μl) was applied to 1% agarose gel-electrophoresis, and we then visualized the bands of 28S and 18S with ethidium bromide under UV light. Band density was measured by light capture (Atto Co., Tokyo, Japan).

Preparation of cDNA and Real-time PCR

Synthesis of complementary DNA (cDNA) from total liver RNA (2 μg) was performed by SuperScript II First-strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed by using "ABI PRISM 7900 Sequence Detection System" (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol in which 5 μl of each cDNA preparation (1/10 dilution) or appropriately diluted standard DNA was added to a 20 μl reaction mixture containing 10 μl of "SYBR Green PCR Master Mix" (Applied Biosystems), 100 nM of each primer and DEPC-water in 0.2 ml MicroAmp optical tubes (Applied Biosystems). The thermal cycling condition comprised initial denaturation (at 95°C for 10 min) and extension (40 cycles at 95°C for 15 sec and 60°C for 1 min). Copies of β-actin mRNA were quantified and presented as copy number per total RNA (μg). Standard DNA and primer sets for β-actin were prepared according to Kasahara *et al.* (2005).

Statistical analysis

Parametric data were analyzed by the F test followed by the Student's *t*-test (Snedecor and Cochran, 1989). A value of $p<0.01$ or 0.05 was considered to be statistically significant.

RESULTS

Effects of conditions (temperature and period) of fixing before freezing the sample

According to the manufacturer's protocol, the tis-

sues were to be fixed in RNAlater® solution before storage. We varied the time and temperature, i.e., rat liver tissues were soaked in RNAlater® and kept for 1, 3, 8, and 15 days under room temperature or in a refrigerator (4°C), and the quality of RNA was checked (Fig. 1). The total RNA yield and OD260/280 ratio of the samples soaked in RNAlater® and kept under room temperature or in a refrigerator were unchanged for the entire period from the first day, whereas the 28S/18S ratio of the sample kept under room temperature was

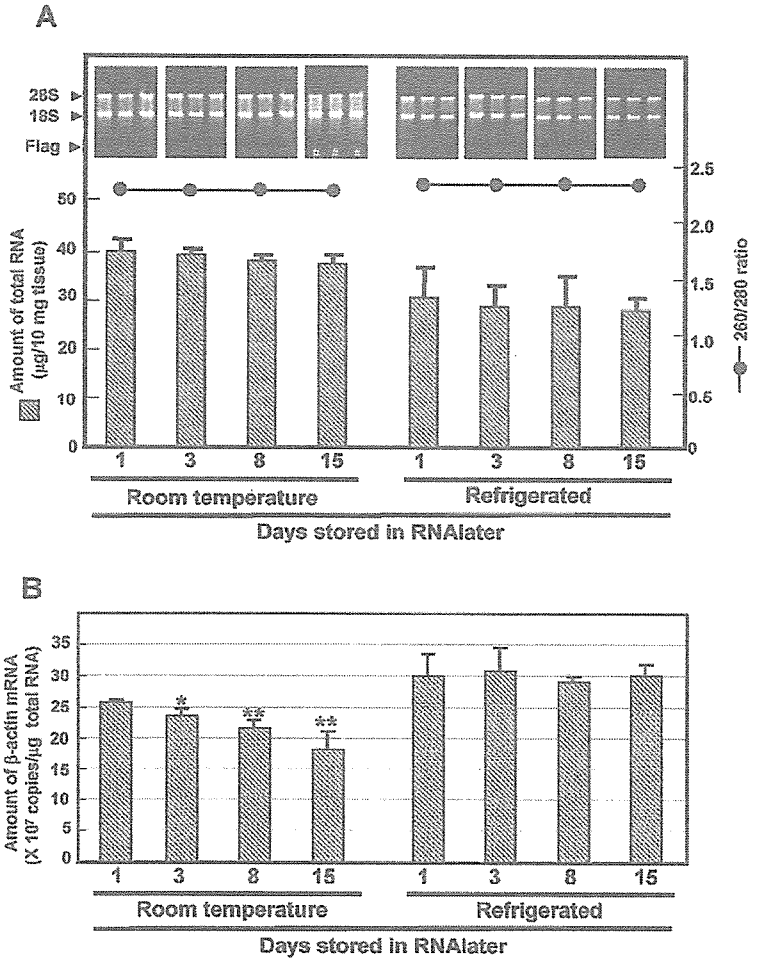

Fig. 1. Influence of temperature and time on RNA quality of rat liver in RNAlater®.
A: Rat liver tissue was soaked in RNAlater® for 1, 3, 8 or 15 days under room temperature or in a refrigerator (at 4°C). Total RNA was extracted from each tissue, and the amount of total RNA (bar braph) and the 260/280 ratio (line graph) were calculated and expressed as the mean ± SD (n=3). Total RNA was subjected to agarose gel-electrophoresis (upper photos), band density of 28S and 18S was measured by densitometer, and the ratio of the 28S/18S band density was calculated. The Flags indicate the degradation of RNA with the following; #; 0.8 ~ 1, ##; ~ 0.8. B: Real-time PCR of β-actin was performed using cDNA synthesized from the total RNA. The amount of expression of β-actin mRNA was expressed as copy number per 1 μg of total RNA. *, **Significantly different from the sample at 1 day by Student's t-test: *$p<0.05$;**$p<0.01$.

found to be abnormal at the 15th day (Fig. 1A). The amount of β-actin mRNA in samples kept in the refrigerator was unchanged throughout the experimental period, whereas this parameter significantly decreased in samples kept for 3 days or more at room temperature (Fig. 1B).

Long-term preservation of samples fixed with RNAlater® 4°C overnight compared with the traditional procedure (liquid N_2 or freezers)

In the next experiment, isolated liver tissues were soaked in RNAlater®, stored overnight in the refrigerator, and then kept at −80 or −20°C, or at −80°C after removing RNAlater®. As a comparison, isolated tissues were frozen in liquid N_2 and kept in liquid N_2 or in a freezer at −20 or −80°C. Their RNA quality was checked at 1, 7 days and 1, 3, 6, and 12 months later. A comparison was made against the sample kept at −80°C with RNAlater® for each time point. These protocols and the results are summarized in Tables 1 - 4.

At one day, the only detectable change was found in the total RNA yield of fresh sample stored at−20°C, and the other parameters were unchanged. At the 7-day stock, the total yield of RNA from the fresh sample stored in liquid N_2 and the sample stored in RNAlater® at −20°C was significantly reduced (Fig. 2-II, C, D; Table 1). Abnormal S28/S18 ratios were observed in one out of 4 fresh samples stored at −80°C and in all 4 samples stored at −20°C. Obvious degradation of RNA was observed in all the fresh samples stored for 7 days at −20°C (Fig. 2-II, F; Table 3), so further preservation was ceased at that point.

At one-month stock, fresh samples stored in liquid N_2 or at −80°C showed significant reduction in the RNA yield with normal S28/S18 ratio (Fig. 2-III, D, E; Table 1, 3). The amount of β-actin mRNA was significantly decreased in the fresh sample stored at −80°C, whereas it increased in the sample RNAlater®-pretreated, stored at −80°C, soaked in RNAlater® and stored at −80°C (Fig. 2-III, B, C, E; Table 4).

Table 1. Evaluation of the methods with and without RNAlater® for preservation of the total RNA in the tissue.

Total RNA yield	Storage period					
Storage method	1-day	7-days	1-month	3-months	6-months	12-months
(a) RNA*later*®, −80℃	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.
(b) RNA*later*® (removed), −80℃	-----	-----	-----	-----	-----	-----
(c) RNA*later*®, −20℃	-----	↓	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	↓	↓	↓	↓	↓
(e) Raw tissue, −80℃	-----	-----	↓	↓	↓	↓
(f) Raw tissue, −20℃	↓	-----	N.P.	N.P.	N.P.	N.P.

N.E.: not evaluated, -----: no change, ↓: decreased ($p<0.05$) vs. method (a), N.P.: not performed. Storage method (a): Isolated liver tissues were soaked in RNAlater® and stored overnight in the refrigerator, and then kept at −80℃ (in a deep freezer). (b) Liver tissues were treated as (a) but stored at −80℃ after removing RNAlater®. (c) Liver tissues treated as (a) and kept at −20℃ (in a freezer) in RNAlater®. (d) Liver tissues were immediately frozen in liquid N_2 and kept in liquid N_2. (e) Frozen tissues were kept at −20℃ (in a freezer). (f) Frozen tissues were kept at −80℃ (in a deep freezer). On the assumption that method (a) was the best, comparisons were made against (a) at each time point.

Table 2. Evaluation of methods with and without RNAlater® for preservation of the quality of RNA (OD 260/280 ratio) in the tissue.

O.D. 260/280 ratio	Storage period					
Storage method	1-day	7-days	1-month	3-months	6-months	12-months
(a) RNA*later*® −80℃	-----	-----	-----	-----	-----	-----
(b) RNA*later*® (removed), −80℃	-----	-----	-----	-----	-----	-----
(c) RNA*later*® −20℃	-----	-----	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	-----	-----	-----	-----	-----
(e) Raw tissue −80℃	-----	-----	-----	-----	-----	-----
(f) Raw tissue −20℃	-----	-----	N.P.	N.P.	N.P.	N.P.

-----: no change N.P.: not performed. Storage methods (a) – (f) are described in Table 1.

For storage for 3 months, there were significant reductions of total RNA in fresh samples stored in liquid N_2 or at −80°C, and abnormal S28/S18 ratio and significant reduction of β-actin mRNA were observed in fresh samples stored at −80°C as well (Fig. 2-IV, D, E; Table 1, 3, 4). For 6-month storage, both fresh samples stored in liquid N_2 and at −80°C showed significant reduction of total RNA, abnormal S28/S18 ratio, and significant reduction of β-actin mRNA (Fig. 2-V, D, E, Table 1, 3, 4). After 12 months of storage, although obvious degradation of RNA was observed in fresh samples stored in liquid N_2 or at −80°C, the extent was less than that at 6 months (Fig. 2-VI, D, E; Table 1, 3, 4).

Influence of time to processing on stability of RNA in frozen samples

When RNA extraction is performed for vast numbers of frozen samples at once, the stability of RNA may be affected since the thawing time is long and varied. In this experiment, fresh samples frozen immediately in liquid N_2 or samples treated with RNAlater® and then frozen were returned to room temperature for various times, and the quality of extracted RNA was checked. In fresh samples, no abnormality in total RNA and OD260/280 ratio was observed at any time point, whereas abnormal 28S/18S ratios were observed at 20 min or later, and significant reduction of β-actin was observed at 10 min or later (Fig. 3, upper panels). On the other hand, samples treated with RNAlater® at 4°C overnight and stored at −80°C did not show any signs of RNA degradation throughout the period of the 2 hr experiments (Fig. 3, lower panels).

Preservation of RNA in RLT reagent

In our project, an RNeasy mini kit (Qiagen) is used for RNA extraction. The stability of RNA in tissue dissolved by Buffer RLT (contained in the kit) was examined under various conditions (Fig. 4). At one-day storage, no significant changes of total RNA and OD260/280 ratio were observed for any conditions, whereas abnormal 28S/18S ratio and significant reduction of β-actin mRNA was observed in samples stored at room temperature (Fig. 4-I, E). For the 7-day stor-

Table 3. Evaluation of methods with and without RNAlater® for preservation of the quality of RNA (28S/18S ratio on RNA electrophoresis) in the tissue.

28S/18S ratio	Storage period					
Storage method	1-day	7-days	1-month	3-months	6-months	12-months
(a) RNA*later*®, −80℃	-----	-----	-----	-----	-----	-----
(b) RNA*later*® (removed), −80℃	-----	-----	-----	-----	-----	-----
(c) RNA*later*®, −20℃	-----	-----	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	-----	-----	-----	*	-----
(e) Raw tissue, −80℃	-----	*	-----	*	*	-----
(f) Raw tissue, −20℃	-----	**	N.P.	N.P.	N.P.	N.P.

-----: no change, *: 28S/18S<1 in a few samples, **: 28S/18S<1 in all samples, N.P.: not performed. Storage methods (a) – (f) are described in Table 1.

Table 4. Evaluation of methods with and without RNAlater® for preservation of β-actin mRNA quantified by Real-time PCR.

Amount of β-actin mRNA	Storage period					
Storage method	1-day	7-day	1-month	3-month	6-month	12-month
(a) RNA*later*®, −80℃	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.
(b) RNA*later*® (removed), −80℃	-----.	-----	-----	-----	-----	-----
(c) RNA*later*®, −20℃	-----	-----	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	-----	-----	-----	↓	↓
(e) Raw tissue, −80℃	-----	↓	↓	↓	↓	-----
(f) Raw tissue, −20℃	-----	↓	N.P.	N.P.	N.P.	N.P.

N.E.: not evaluated, -----: no change, ↓: decreased ($p<0.05$) vs. method (a), N.P.: not performed. Storage methods (a) – (f) are described in Table 1. On the assumption that the method (a) was the best, comparisons were made against (a) at each time point.

age, an abnormal 28S/18S ratio was observed both in samples stored at room temperature and at 4°C (Fig. 4-II, D,E). Samples stored at room temperature also showed significant reduction in β-actin mRNA (Fig. 4-II, E). Obvious signs of RNA degradation were observed at 7 days in samples stored at room temperature and 4°C, so further examinations were not performed under these conditions.

In samples stored at −20 and −80°C for one month, a significant increase in RNA yield was observed, whereas no abnormalities were noted in the OD260/280 or 20S/18S ratios (Fig. 4-III, B, C). In samples stored for 3 months, no significant changes or abnormalities were detected for any conditions (Fig. 4-IV). In samples stored for 6 months, no changes were observed in any conditions except that the samples stored at −20°C showed a significant decrease in β-actin mRNA (Fig. 4-V, C). For samples stored for 12 months, the samples stored at −20°C showed an abnormal 28S/18S ratio and significant decrease in β-actin mRNA (Fig.4-VI, C).

DISCUSSION

The aim of our project is to create a database of transcriptome that is appropriate for prediction of drug toxicity in the early stage of drug development. It is therefore quite important to obtain gene expression data with high accuracy, and subsequently the method of preservation of the samples and the RNA extraction procedure are highly important. In the present study, the quality of RNA was checked in terms of the following 4 measures: amount of β-actin mRNA, 28S/18S ratio of RNA on agarose electrophoresis, yield of total RNA, and OD260/280 ratio. The amount of β-actin mRNA showed a good correlation with the 28S/18S ratio and appeared to be more sensitive than the latter: namely, β-actin was decreased earlier than when the

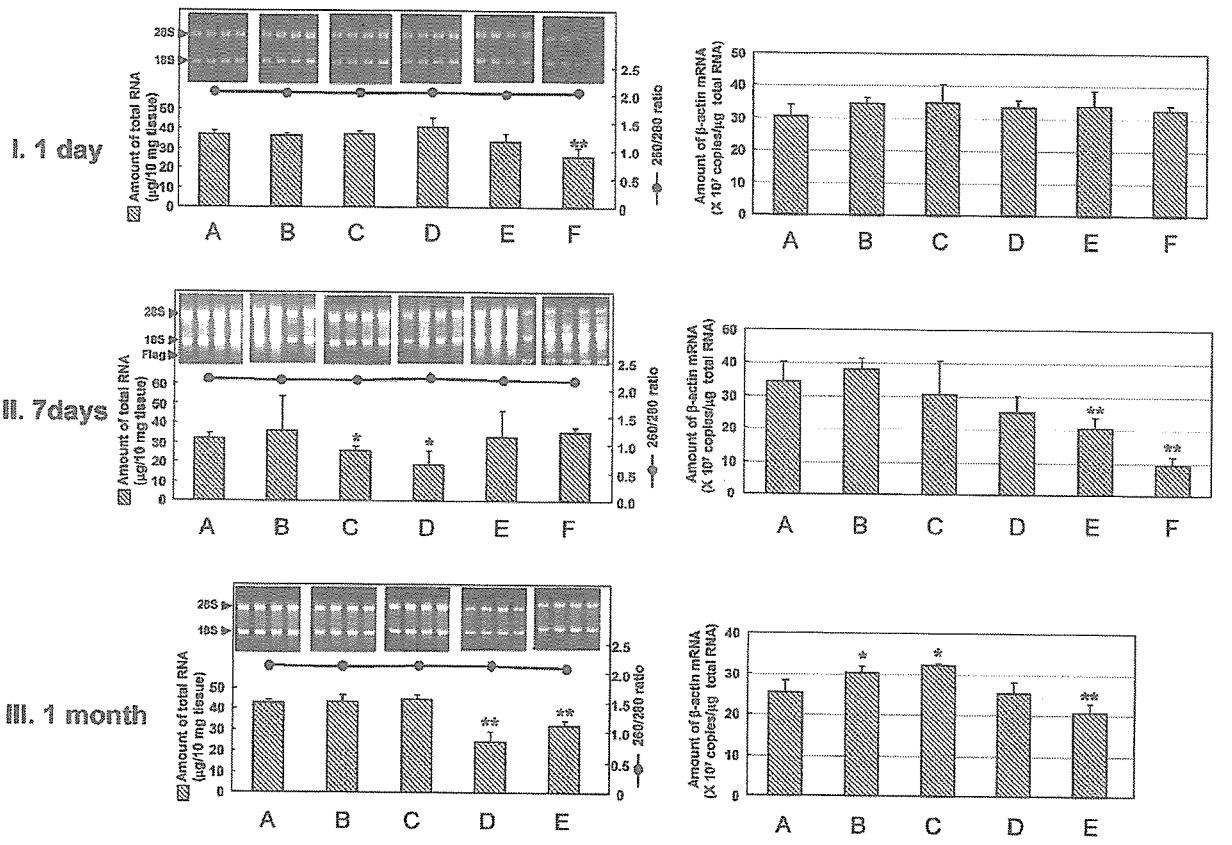

Fig. 2-1.

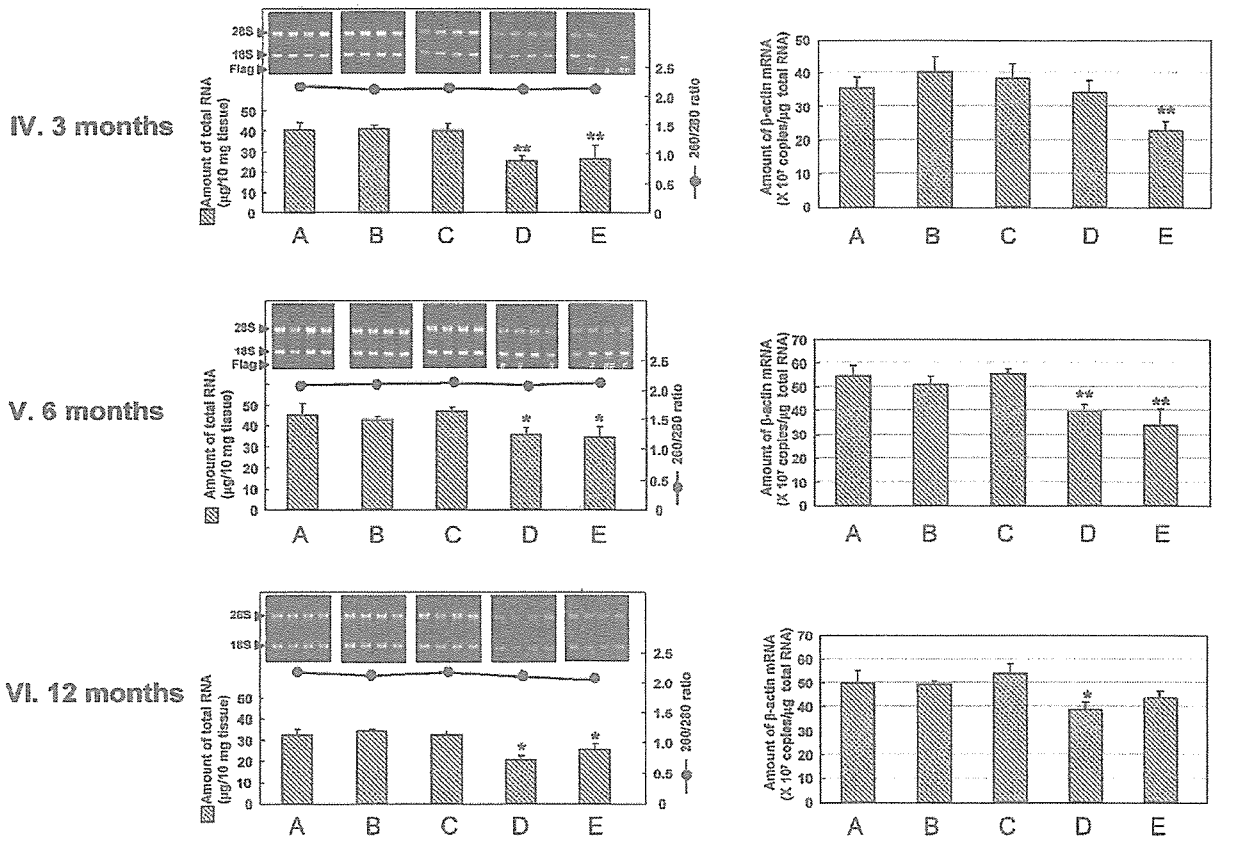

Fig. 2-2. Comparison of the methods with and without RNAlater® for preservation of tissue in terms of quality of RNA. Rat liver tissue was soaked in RNAlater® overnight at 4°C, and stored under the following conditions: A: at −80°C with RNAlater®, B: at −80°C after removing RNAlater®, C: at −20°C with RNAlater®. Raw tissues without treatment with RNAlater® were stored under the following conditions: D: in liquid nitrogen, E: at −80°C, F: at −20°C. Total RNA was extracted at 1 day, 7 days, one month, 3 months, 6 months and 12 months after storage. Left panels: Total RNA was extracted from each tissue, and the amount of total RNA (bar graph) and the 260/280 ratio (line graph) were calculated and expressed as the mean± SD (n=4). Total RNA was subjected to agarose gel-electrophoresis (upper photos), band density of 28S and 18S was measured by densitometer, and the ratio of the 28S/18S band density was calculated. The Flags indicate the degradation of RNA with the following; #; 0.8 ~ 1, ##; ~ 0.8. Right panels: Real-time PCR of β-actin was performed using cDNA synthesized from the total RNA. The amount of expression of β-actin mRNA was expressed as copy number per 1 μg of total RNA. *, **Significantly different from the sample at 1 day by Student's t-test: *$p<0.05$;**$p<0.01$. Raw tissue stored at −20°C (F) was not examined later than 7 days, because it showed obvious signs of RNA degradation at the 7th day.

abnormal ratio of 28S/18S ratio was detected. The total yield of RNA and OD260/280 ratio were not correlated with the 28S/18S ratio. This could be due to employment of the column method for extraction of RNA where degraded short RNA chains were also harvested. The OD 260/280 ratio is an index of the contamination of proteins and thus showed no abnormalities in the case of RNA degradation.

Whether the normal range of these parameters assures the quality of RNA for GeneChip analysis is another question. It is well known that the stability of RNA varies largely between genes (Timofeeva *et al.*, 2000), and degradation of RNA differentially affects each probe set for a gene (Thach *et al.*, 2003). Therefore, the present study does not necessarily assure quantification of each gene expression. However, it is not practical to perform GeneChip analyses for all the conditions in the present study in order to assess the

individual stability of all the genes. Here we offer the minimal requirement for construction of the database for Toxicogenomics.

According to the manufacturer's protocol, the samples are to be preserved by RNAlater® for 1 week under room temperature and for 4 weeks under 4°C. Similar results were obtained in our laboratory. As for the amount of β-actin mRNA in the sample stored with RNAlater® at room temperature, some reduction was observed at 3 days and obvious reduction was detected at 8 days, whereas no reduction of RNA quality was detected under 4°C as long as 15 days. Therefore, we concluded that the sample soaked in RNAlater® could be stored at room temperature for a few days, but preferentially should be stored in a refrigerator which would make it possible to keep it for at least 2 weeks.

The manufacturer's protocol recommends removing RNAlater® after overnight treatment when preserved in the freezer. In our project, however, we harvested 160 samples (N=5 for each of 4 time and 4 dose points in a duplicated manner) per experiment, but 48 of them (N=3 for each of 4 time and 4 dose points) were analyzed by GeneChip. This means only 30% of the tubes are homogenized and the remaining ones are stored for backup, so it is quite cumbersome if RNAlater® is to be removed from all the tubes. We compared RNA quality between samples frozen with and without RNAlater® and no difference was observed. It appears that RNAlater® can be left during storage in the frozen state, at least in the case of liver tissue.

For samples stored in liquid N_2 or at −80°C, some signs suggesting RNA degradation appeared until 6 months, whereas degradation tended to reduce by 12 months. This irrational result might be explained as follows. In the experiments shown in Fig. 2, the samples should have been thawed before homogenization, and this took 5 to 10 min because there were so many tubes. The total number of the samples was small when the 12-month samples were processed, and thus the time for processing was shorter than the other time points. This suggested that the time from thawing to

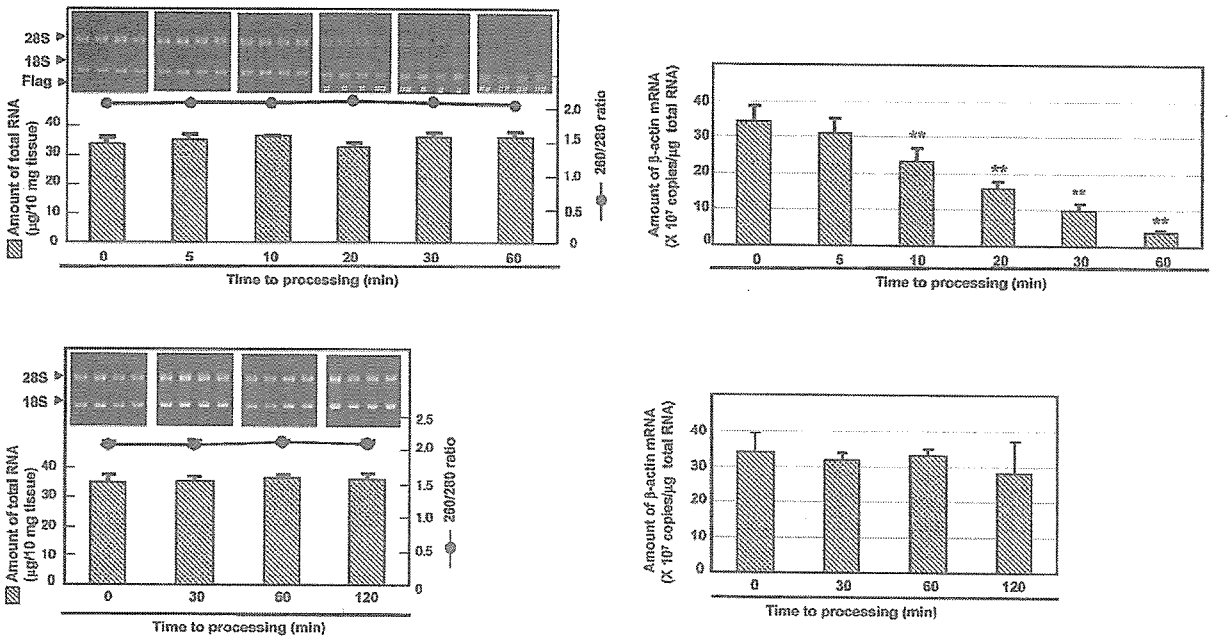

Fig. 3. Effects of freezing-thawing on the RNA quality in the sample.
Upper panels: Non-treated samples were taken out from liquid nitrogen and left for 0, 5, 10, 20, 30 or 60 min under room temperature and then lysed in buffer RLT.
Lower panels: RNAlater®-fixed samples were taken out from the deep freezer and left for 0, 30, 60, or 120 min under room temperature and then lysed in buffer RLT. Checking of RNA quality (left panels) and quantification of β-actin mRNA by real-time PCR (right panels) were performed in the same way as in Fig 2.

homogenization affected RNA quality. In order to confirm this, we compared these samples: frozen without preservative in liquid N_2 and frozen at −80°C after RNAlater®-treatment by leaving them to thaw. In the samples without RNAlater®, signs of RNA degradation appeared at 10 min or later, whereas RNA appeared to be stable in the RNAlater®-treated samples for at least 2 hrs. It also appeared that degradation of RNA was more dependent on the time of thawing than that of storage when stored without preservatives. This clearly means that storage in RNAlater® has a merit in processing many frozen samples at once without concern for RNA degradation. The manufacturer's note points out that RNAlater®-treated samples can be repeated in the freezing-thawing cycle up to 10 times. We also confirmed this description (data not shown), although freezing-thawing is restricted to once in our general protocol.

The manufacturer's note also describes that RNAlater® is useful in preserving cultured cells, i.e., cells are scraped from the plates, harvested by centrifugation and RNAlater® added to the pellet. However, this procedure is quite cumbersome and might affect gene expression in the cell. In our project, we directly dissolved the cells by Buffer RLT Reagent, a component of the RNA-extraction kit. It would be quite convenient if the RNA in the dissolved sample were stable under this condition. The present experiments using liver tissues demonstrated that the RNA in samples dissolved in the buffer appeared to be stable for at least one year at −80°C or in liquid N_2.

From the experiments described above, we offer

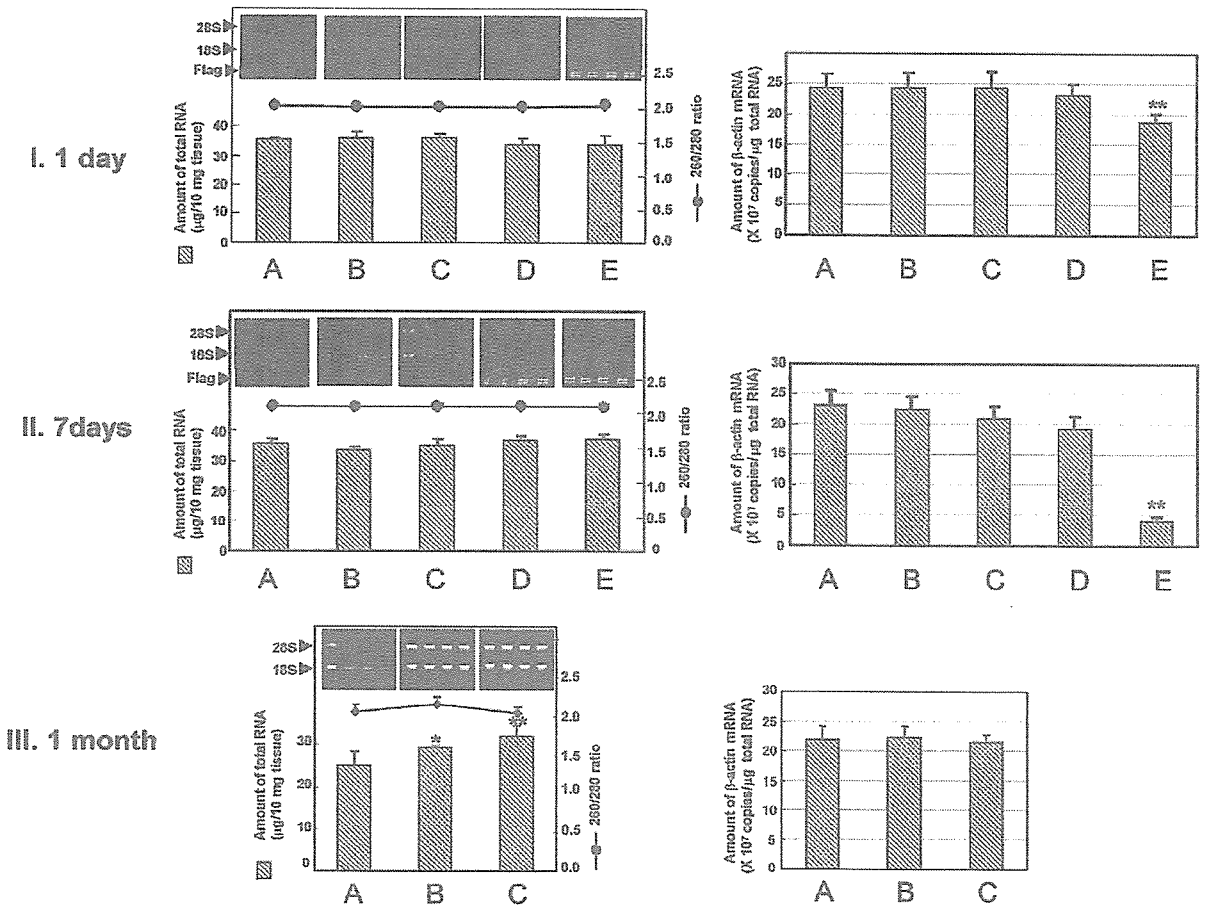

Fig. 4-1.

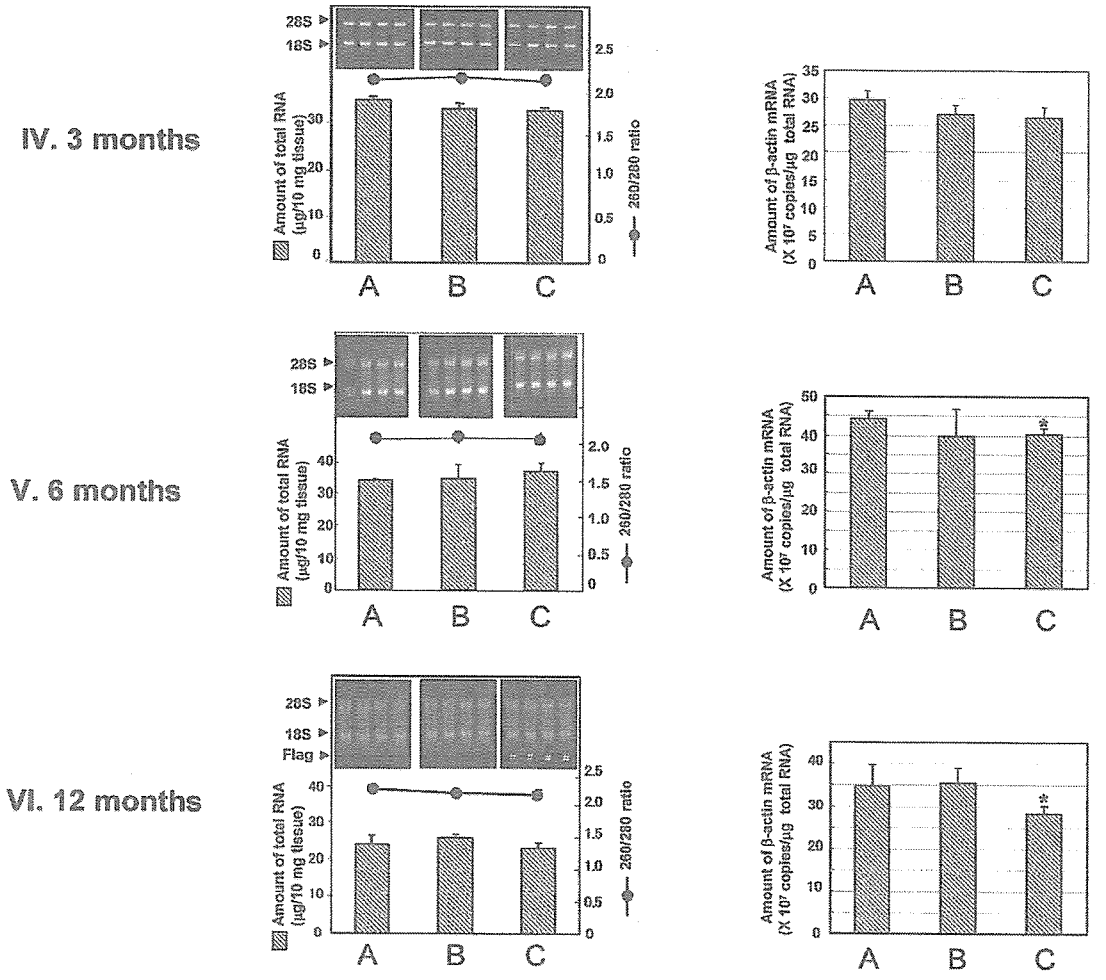

Fig. 4-2. Evaluation on stability of RNA in the sample dissolved in Buffer RLT. Rat liver tissue was dissolved in buffer RLT. The liver lysate, 100 μl each, was dispensed to microtubes and stored under the following conditions: A: in liquid nitrogen, B: −80°C, C: −20°C, D: 4°C, E: room temperature. Total RNA was extracted 1 day, 7 days, one month, 3 months, 6 months and 12 months after storage. Checking of RNA quality (left panels) and quantification of β-actin mRNA by real-time PCR (right panels) were performed in the same way as in Fig 2. The lysates stored at −20°C and room temperature (D, E) were not examined later than 7 days, because they showed obvious signs of RNA degradation at the 7th day.

the following protocol as a minimum requirement for RNA extraction from rat liver for GeneChip analysis.

1. Liver tissues are to be dissected into 5 mm cubic pieces on ice and put into a tube containing more than 10 volumes of chilled RNAlater® as soon as possible. A disposable biopsy punch is useful for this purpose.
2. The tissue samples are to be fixed in RNAlater® overnight at 4°C. It can be stored at least one day at room temperature, and 2 weeks at at 4°C, in the non-frozen state.
3. For longer storage of RNAlater®-fixed samples, they should be kept under −20°C and are stable for at least one year. For this purpose, RNAlater® is not necessarily removed.
4. When RNA is extracted from samples treated with RNAlater® and frozen for storage, they should be homogenized with Buffer RLT within 2 hr.
5. The samples dissolved in Buffer RLT can be stored for at least one year without RNA degrada-

tion at −80°C.

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Effect of the difference in vehicles on gene expression in the rat liver—analysis of the control data in the Toxicogenomics Project Database

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Abstract

The Toxicogenomics Project is a 5-year collaborative project by the Japanese government and pharmaceutical companies in 2002. Its aim is to construct a large-scale toxicology database of 150 compounds orally administered to rats. The test consists of a single administration test (3, 6, 9 and 24 h) and a repeated administration test (3, 7, 14 and 28 days), and the conventional toxicology data together with the gene expression data in liver as analyzed by using Affymetrix GeneChip are being accumulated. In the project, either methylcellulose or corn oil is employed as vehicle. We examined whether the vehicle itself affects the analysis of gene expression and found that corn oil alone affected the food consumption and biochemical parameters mainly related to lipid metabolism, and this accompanied typical changes in the gene expression. Most of the genes modulated by corn oil were related to cholesterol or fatty acid metabolism (e.g., CYP7A1, CYP8B1, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, squalene epoxidase, angiopoietin-like protein 4, fatty acid synthase, fatty acid binding proteins), suggesting that the response was physiologic to the oil intake. Many of the lipid-related genes showed circadian rhythm within a day, but the expression pattern of general clock genes (e.g., period 2, arylhydrocarbon nuclear receptor translocator-like, D site albumin promoter binding protein) were unaffected by corn oil, suggesting that the effects are specific for lipid metabolism. These results would be useful for usage of the database especially when drugs with different vehicle control are compared.

Keywords: Toxicogenomics; Vehicle control; Methylcellulose; Corn oil; Lipid metabolism; Rat; Liver

Introduction

The Toxicogenomics Project is a 5-year collaborative project by the National Institute of Health Sciences (NIHS) and 17 pharmaceutical companies in Japan which started in 2002 (Urushidani and Nagao, 2005). In April 2005, some rearrangements were made and now the project is conducted by NIHS, the National Institute of Biomedical Innovation, and 16 pharmaceutical companies. Its aim is to construct a large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medicinal compounds, have been selected, and the following are examined for each. The in vivo test using rat consists of a single administration test (3, 6, 9 and 24 h with 4 dose levels including vehicle control) as well as a repeated administration test (3, 7, 14 and 28 days with 4 dose levels including vehicle control), and the data of body weight, general symptoms, histopathological examination of liver and kidney, and blood biochemistry are obtained from each animal. The gene expression in liver (and kidney in some cases) is comprehensively analyzed by using Affymetrix GeneChip. An in vitro test using rat and human hepatocytes is also carried out to accomplish the bridging between the species. By April 2005, more than 100 chemicals, covering wide medication categories, have been finished or are ongoing.

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Along with the effects of the chemicals, a vast amount of control data is being accumulated.

The main purpose of the project is to predict toxicity in the early stage of drug development. The potential usefulness of microarray data for the estimation of toxicity of drugs makes it possible for this technology to be used in the late stage of development, i.e., application in the field of regulatory science. In this case, however, more strict and precise validation is needed in order to assure the reliability of the data. It is well known that a difference in the platform considerably effects a variation in the microarray data (Waring et al., 2004) and this is quite difficult to overcome. In our project, either methylcellulose or corn oil is employed as vehicle, according to the dispensability of the drug. It is quite possible that the difference in the vehicle control affects the analysis, as observed by multiple comparison of drug effects. In traditional toxicological study, comparison of the drug is exclusively made against its vehicle control. However, in the transcriptome database, it is usually necessary to make a comparison among various drugs by clustering or discriminant analysis. The history of this field is not old enough for collecting appropriate data regarding this issue. Our database enables us to make various comparisons among different vehicles, protocols, facilities, chip versions, etc. In this present report, we focus on the influence of vehicles on the control parameters including the gene expression profile in the rat liver as a basic study for future analysis.

Materials and methods

Animal treatment

Male Sprague-Dawley rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 animals using a computerized stratified random grouping method based on the body weight for each age. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 h (7:00–19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21–25 °C with a relative humidity of 40–70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Japan).

According to the protocol in our project, rats in each group were orally administered with various drugs suspended or dissolved either in 0.5% methylcellulose solution or corn oil according to their dispersibility. Each drug had 4 different dose levels, including the vehicle control alone, which was exclusively analyzed in the present study. Drug treatment was performed between 9:00 and 11:30 a.m. For single-dose experiments, rats were sacrificed at 3, 6, 9, and 24 h after dosing. For repeated dose experiments, the animals were treated for 3, 7, 14 or 28 days, and they were sacrificed 24 h after the last dosing. Body weights were recorded every day while food consumption was recorded every 4 days during repeated dosing. Blood samples were collected upon sacrifice in tubes containing heparin lithium (blood biochemistry), EDTA-2K (hematology), or 1/9 vol of 3.8% citric acid (coagulation), and the following items were examined: hematology: the numbers of red blood cells, reticulocytes, white blood cells, eosinophils, monocytes, platelets, neutrophils, basophils, and lymphocytes, hemoglobin, mean red blood cell volume, mean hemoglobin contents, and mean hemoglobin concentration (Advia 120, Bayer); blood coagulation: prothorombin time, active partial prothrombin time, and fibrinogen (Sysmex CA-5000, Sysmex); and blood biochemistry: alkaline phosphatase, total cholesterol, triglyceride, phospholipid, total and direct bilirubin, glucose, blood urea nitrogen, creatinine, Na, K, Ca, Cl, inorganic phosphate, total protein, albumin, globulin/albumin ratio, aspartate aminoptansferase, alanine aminotransferase, lactate dehydrogenase, and γ-glutamyltranspeptidase, which were determined by an autoanalyzer (Hitachi 7080).

When the analysis was performed (April 2005), 65 compounds had been completed in 4 different contract research organizations. In order to eliminate the variations due to the difference in the facility, we selected a laboratory (Japan Bioassay Center, Kanagawa, Japan) where at least 7 experiments for each vehicle were completed. As 10 experiments were done with methylcellulose as the vehicle there, the latest 3 of them were excluded from the present analysis to match the numbers. Therefore, each time point consists of 35 (5 rats for 7 experiments) animals.

The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of National Institute of Health Sciences.

Microarray analysis

After collecting the blood, the animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia. An aliquot of the sample (about 30 mg) for RNA analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNA later® (Ambion, Austin, TX, USA) overnight at 4 °C, and then frozen to send to the facility in the National Institute of Health Sciences.

Total RNA was isolated using RNeasy kit by Bio Robot 3000 (Qiagen, Valencia, CA, USA). Homogenization was conducted by Mill Mixer (Qiagen) and zirconium beads. Purity of the RNA was checked by gel electrophoresis confirming the 260/280 nm ratio was between 2.0 and 2.2.

Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip® RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA), containing 15923 probe sets. The procedure was conducted basically according to the manufacturer's instructions using Superscript Choice System (Invitrogen, Carlsbad, CA, USA) and T7-$(dT)_{24}$-oligonucleotide primer (Affymetrix) for cDNA synthesis, cDNA Cleanup Module (Affymetrix) for purification, and BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA) for synthesis of biotin-labeled cRNA. Ten micrograms of fragmented cRNA was hybridized to a RAE230A probe array for 18 h at 45 °C at 60 rpm, after which the array was washed and stained by streptavidin-phycoerythrin using