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Research Article

Expression of Genes Encoding for Xenobiotic Metabolism After Exposure to Dialkylnitrosamines in the Chicken Egg Genotoxicity Alternative Model

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ABSTRACT

The Chicken Egg Genotoxicity Assay (CEGA) demonstrated responsiveness to various DNA-reactive chemicals requiring metabolic activation, which implies broad bioactivation capability. To assess potential metabolic competence, expression profiles of metabolic genes in the embryo-chicken fetal liver were determined using microarray technology. Fertilized chicken eggs were injected under the CEGA protocol with vehicle (deionized water [DW]), the activation-dependent carcinogens, diethylnitrosamine (DEN), and *N*-nitrosodiethanolamine (NDELA) at doses producing no effect on survival. Previously in CEGA, DEN produced DNA damage, whereas NDELA did not. Expressions of 463 genes known to encode for phase I and II of endo- and xenobiotic metabolism were detected on the array. DW did not affect the expression of the selected genes, deregulating less than 1% of them. In contrast, DEN at 2 mg/egg and NDELA at 4 mg/egg produced significant transcriptomic alterations, up-regulating up to 41% and down-regulating over 31% of studied genes. Both nitrosamines modulated the majority of the genes in a similar manner, sharing 64 up-regulated and 93 down-regulated genes with respect to control group, indicating similarity in the regulation of their metabolism by avian liver. Differences in gene expression between DEN and NDELA were documented for several phase I CYP 450 genes that are responsible for nitrosamine biotransformation, as well as for phase II genes that regulate detoxication reactions. These findings could underlie the difference in genotoxicity of DEN and NDELA in CEGA. In conclusion, the analysis of gene expression profiles in embryo-chicken fetal liver dosed with dialkylnitrosamines demonstrated that avian species possess a complex array of inducible genes coding for biotransformation.

Key words: Chicken Egg Genotoxicity Assay; gene expression profile; genes involved in xenobiotic metabolism; microarray; nitrosamines.

The Chicken Egg Genotoxicity Assay

The Chicken Egg Genotoxicity Assay (CEGA) and the related Turkey Egg Genotoxicity Assay (TEGA) are novel alternatives to animal models, which have been used for the screening of genotoxic potential of a variety of chemicals (Iatropoulos et al., 2017; Kobets et al., 2016, 2018; Williams et al., 2011a, 2014). TEGA and CEGA were developed as potential follow-up assays to the existing regulatory *in vitro* assays in an effort to minimize the use of rodent assays. Among important aspects of the *in ovo* model is the utilization of an intact organism that is not

considered to be a live animal in compliance with Animals (Scientific Procedures) Act 1986, since the termination in CEGA occurs 10–11 days before hatching. Additionally, the nervous system of the avian embryo is not finally formed by the termination time point (Hughes, 1953), so potential discomfort to the organism during the procedure and termination does not occur. Thus, CEGA provides a useful replacement for *in vivo* genotoxicity assessment where the use of animals is undesirable or precluded. Rigorous CEGA protocol precludes many artifacts, including influences of numerous environmental factors, which is difficult to accomplish in other mature experimental animals.

Currently, CEGA is the only nonanimal model that allows for an extensive evaluation of multiple critical endpoints in the avian fetal liver indicative of potential genotoxicity, namely the comet assay for detection of DNA strand breaks (Ostling and Johanson, 1984; Singh et al., 1988; Tice et al., 2000), and the nucleotide ^{32}P-postlabeling assay for DNA adducts detection (Phillips and Arlt, 2014; Randerath et al., 1981). Also, the model allows assessment of other critical endpoints, including biotransformation activities (Perrone et al., 2004), cell proliferation (unpublished), histopathologic evaluation (Iatropoulos et al., 2017), and as reported here, transcriptomic analysis.

The genotoxic effects of chemicals in CEGA and TEGA are similar to those *in vivo* (Iatropoulos et al., 2017; Kobets et al., 2016, 2018; Williams et al., 2011a, 2014), which reflects the fact that the development and histopathological structure of the avian liver resembles that of rodents and humans (Golbar et al., 2012; Iatropoulos et al., 2017; Ross and Pawlina, 2006; Yokouchi, 2005). Moreover, avian fetal liver in early stages of development is involved in all of the metabolic processes required to sustain the developing autonomous organism (Lorr and Bloom, 1987; Sinclair and Sinclair, 1993). Thus, avian fetal liver expresses major phase I and phase II biotransformation enzymes (Hamilton et al., 1983; Ignarro and Shideman, 1968; Jackson et al., 1986; Perrone et al., 2004; Rifkind et al., 1979, 1994; Sinclair and Sinclair, 1993; Wolf and Luepke, 1997), activity of which is comparable to that in postnatal rodents (Perrone et al., 2004). These enzymes often play a major role in bioactivation of genotoxic chemical carcinogens, thereby making CEGA and TEGA attractive models for detecting effects of activation-dependent carcinogens without using an exogenous source of enzymes. Moreover, mimicking *in vivo* models, the avian embryo-fetus is capable of detoxication and elimination of xenobiotics, in contrast to *in vitro* systems (Perrone et al., 2004; Romanoff, 1960; Wolf and Luepke, 1997).

Additionally, CEGA is the first alternative genotoxicity model, which also allows analysis of tissue-specific gene expression, since it utilizes the liver of an intact organism as opposed to *in vitro* methods. A highly specific and sensitive, validated high-throughput microarray platform, allows the study of chicken functional genomics (Li et al., 2008). Similar to other vertebrates, approximately 35 000 distinct transcripts were identified in chicken, almost 40% of which have orthologs in other organisms (Boardman et al., 2002). Moreover, published literature provides proof of the positive correlation between gene expression patterns in chicken and other vertebrates (Nie et al., 2010), providing additional evidence that avian species can be utilized along with rodent models. While the presence of liver-specific endo- and xenobiotic-related genes has been previously described in chicken (Li et al., 2009), the modulation of their expression in response to xenobiotic exposure has not been investigated in detail.

Accordingly, in the current study, gene expression data from chicken fetal liver were analyzed for the presence and expression of genes that code for enzymes involved in the metabolism of endo- and xenobiotics. Gene activity was assessed following repeat administration under CEGA conditions of two activation-dependent carcinogenic *N*-nitrosamines, diethylnitrosamine (DEN), and *N*-nitrosodiethanolamine (NDELA), compared with the control group injected with vehicle (DW). Data were also obtained on other genes present on the platform, but the current report focuses only on the findings for the expression of metabolic genes in order to support the proficiency of avian embryo-fetal liver in chemical biotransformation.

N-*Nitrosamines*

Nitrosamines have been extensively evaluated in various animal species, revealing that the majority of compounds from this group produced sufficient evidence for carcinogenicity in laboratory animals. Both, DEN and NDELA in experimental animals induce tumors in multiple sites, including liver (IARC, 1978, 2000; Lijinsky, 1987).

These nitrosamines were previously evaluated in CEGA for their genotoxic potential, revealing that DEN produced DNA damage, whereas NDELA did not (Williams et al., 2014). Similar results were obtained in another *in ovo* model, hen's egg micronucleus assay, which assesses mutagenic properties of chemical agents (Wolf et al., 2003). Phenotypic changes produced by these chemicals in the chicken fetal liver were congruent with the molecular alterations observed in CEGA (Iatropoulos et al., 2017). Specifically, DEN produced dose-related distortion of liver architecture, whereas livers in a group dosed with NDELA resembled those in control groups. In addition, only DEN produced agenesis of the gallbladder in chicken and turkey fetuses (Iatropoulos et al., 2017; Williams et al. 2011b). In rodents, the teratogenic potential of DEN has not been reported, which was attributed to lack of activating enzymes in the fetus (Arcos et al., 1982; IARC, 1978). Table 1 summarizes previous findings of testing DEN and NDELA in CEGA.

Genotoxicity and carcinogenicity of nitrosamines is attributed to their metabolic conversion to form alkylating agents (Lijinsky, 1987). These active metabolites then react with macromolecules, such as RNA and DNA, producing adducts at various sites, eg, O^6 of guanine, O^2 and O^4 of thymidine and uridine, and N^6 of adenosine (Loveless, 1969; Magee, 1971; Swann and Magee, 1968). While DEN, as is the case for most genotoxic nitrosamines, is activated by cytochrome P450-mediated α-hydroxylation of the carbon adjacent to the nitrosamino group, the major metabolic route for NDELA is β-oxidation mediated by alcohol dehydrogenase (ALD) (Figure 1) (Bonfanti et al., 1987; Loeppky, 1999). This difference in metabolic routes can contribute to differences in genotoxic potential of DEN and NDELA (Lijinsky, 1987). Some studies also suggest that sulfation can play a role in activation of NDELA (Sterzel and Eisenbrand, 1986). Denitrosation, which also is mediated by cytochromes, is considered to be a detoxication pathway for nitrosamines, which competes with activation pathways (Hecht, 1997). The major elimination of the nitrosamines occurs in urine either unchanged or conjugated with glucuronide or sulfate (IARC, 1978, 2000).

MATERIALS AND METHODS

Tested chemicals. The chemical structures of the tested compounds are shown in Figure 1. DEN (CAS: 55-18-5; ≥99% pure as reported by the supplier) and NDELA (CAS: 1116-54-7; ≥90%) were purchased from Sigma-Aldrich (St. Louis, Missouri). Deionized water (DW) prepared with a Picopure System (Hydro

Table 1. Previous Findings of Diethylnitrosamine (DEN) and *N*-Nitrosodiethanolamine (NDELA) Testing in CEGA

Compound	Dose Tested*, mg/egg	Assay	Results	References
DEN	0.125–4	Comet	+	Williams et al. (2014)
	0.125–4	NPL	–	Williams et al. (2014)
	1–2	Histopathological evaluation	DEN at 1 mg/egg produced distortion of hepatocellular trabecular pattern as well as severe cholangiocellular anisokaryosis, anisocytosis, and dysplasia. At 2 mg/egg, DEN produced gallbladder agenesis and compensatory ductal metaplasia	Iatropoulos et al. (2017)
NDELA	0.5–4	Comet	–	Williams et al. (2014)
	0.5–4	NPL	–	Williams et al. (2014)
	4	Histopathological evaluation	In groups dosed with 4 mg/egg of NDELA hepatocellular trabecular pattern was intact with resident cell populations and elements of the ECM resembling the morphology of vehicle control group	Iatropoulos et al. (2017)

+, positive; –, negative. *, administered in 3 daily doses on days 9, 10, and 11, with termination on days 11 (comet and NPL) or 12 and 18 (histopathological evaluation). NPL, ^{32}P-nucleotide postlabeling assay.

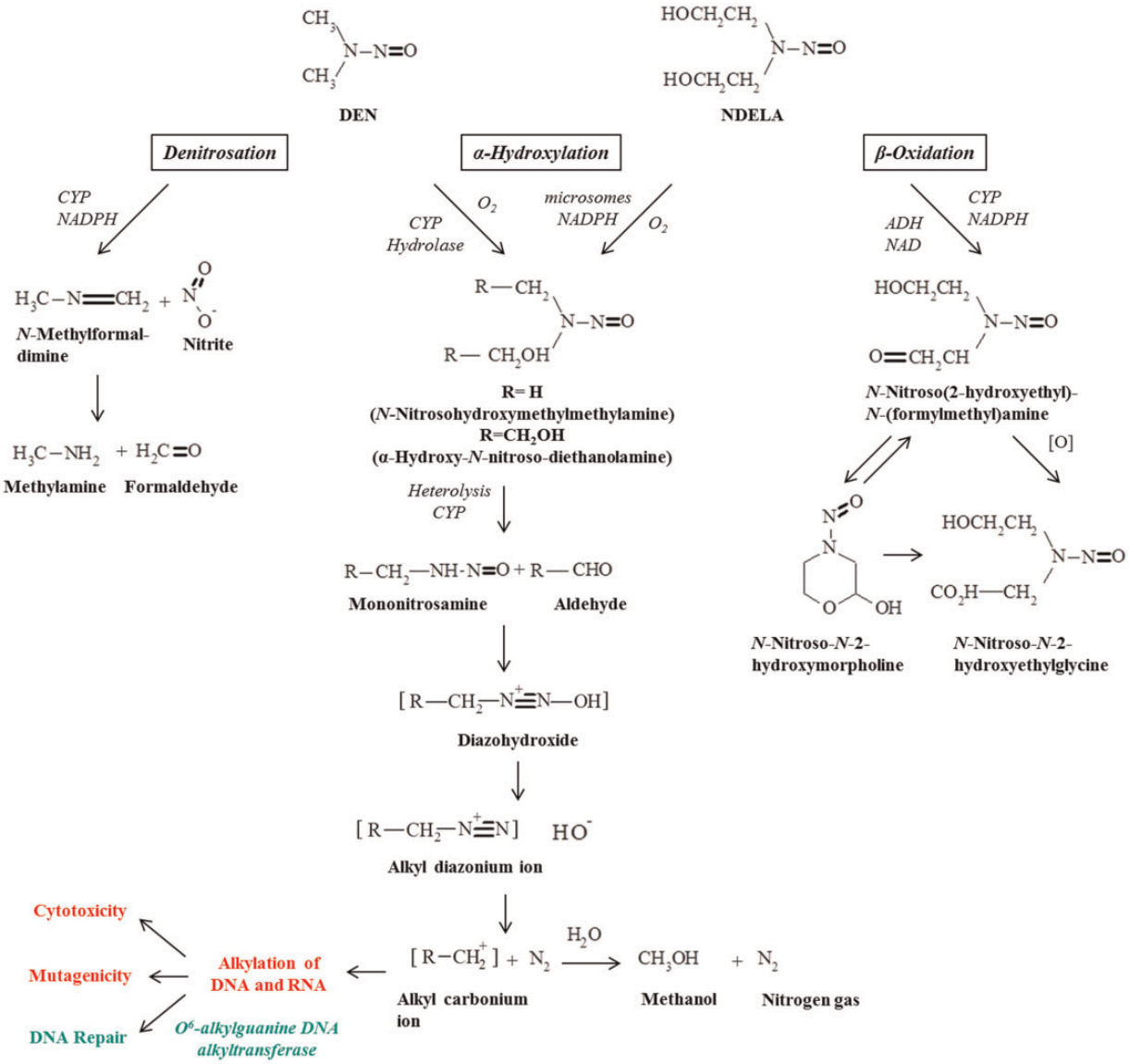

Figure 1. Metabolic conversion of diethylnitrosamine (DEN) and *N*-nitrosodiethanolamine (NDELA). ADH, alcohol dehydrogenase; CYP, cytochrome P450; NAD, nicotinamide-adenine dinucleotide; NADPH, nicotinamide-adenine dinucleotide phosphate (reduced form).

Services and Supplies, Garfield, New Jersey), which has an online resistance (10 MOhm) monitor, was used as a vehicle for both chemicals.

Experimental design. Fertilized specific pathogen-free premium white leghorn chicken (*Gallus gallus*) eggs of undetermined sex were purchased from Charles River (North Franklin, Connecticut). Upon arrival, eggs were numbered, weighed, and randomly divided into control and dosed groups (at least 10 eggs per group). The first day of incubation was designated as Day 0. Eggs were incubated in GQF Manufacturing Company Hova Bator Model 2362N styrofoam incubators (Murray McMurray Hatchery, Webster City, Iowa) with automatic egg turners at $37 \pm 0.5^{\circ}C$ and $60 \pm 5\%$ humidity. Viability was assessed on day 8 by transillumination, eggs that did not develop were eliminated. Control and dosed eggs were separated to avoid cross contamination. Doses of compounds were selected based on the previous findings in CEGA (Williams et al., 2014). The dose selected was the dose that produced genotoxic and morphologic effects but/or did not produce a decrease in viability levels higher than 50% (at least 50% of fetuses in the group are viable upon opening eggs at termination), in order to avoid false positive results due to cytotoxicity. Vehicle (DW) as well as tested compounds, DEN at 2 mg/egg and NDELA at 4 mg/egg, were administered in total volume of 0.15 ml/egg via 3 daily injections into the air sac on days 9 through 11 of incubation. An additional group, environmental control, did not receive any injections. Chicken fetuses were terminated 3 h after the last injection by decapitation. Fetal weights were recorded. Livers were removed and weighed, and frozen at $-80^{\circ}C$ for subsequent gene expression analysis.

RNA extraction. Total RNA was extracted from chicken fetal liver ($n = 4$ liver samples per group per compound) using RNeasy Mini kit (Qiagen, Valencia, California) according to the manufacturer's protocol. The concentration of samples was determined by NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). The quality of total RNA was assessed on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California), and RNA integrity number (RIN) was established to be on average 9.6, ranging from 10 to 8.7 for each sample.

Microarray. About 825 ng of RNA (with RIN $>$ 9.10) was reverse transcribed and labeled with fluorescent tags Cy3/Cy5 dUTP using Low Input Quick Amp Labeling Kit (Agilent Technologies). Microarray processing was performed in 4 biological replicas (4 samples [replicas] per group) as detailed in a previously published protocol (Velíšková et al., 2015) using Agilent 60 whole genome 4X44 chicken V2 microarray platform (Agilent Technologies). The hybridized slides were scanned with an Agilent Dual Laser Scanner G2539A (Agilent Technologies). The resulting images were analyzed using Agilent Feature Extraction 11.1 software. The raw intensity values were normalized using previously published algorithms (Lee et al., 2017).

Gene expression data complying with the "Minimum Information about Microarray Experiments" (MIAME) have been made available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) as series GSE110904 and GSE110906.

Statistical and bioinformatic analysis of microarray data. Genes were considered as regulated if their absolute fold-change exceeded the pool estimate of biological variability and technical noise (Iacobas et al., 2018). The regulation was considered as significant if the p-value of the paired t-test with Bonferroni correction for multiple testing was less than 0.05 (Iacobas et al., 2007). The similarity SIM between the transcriptomic effects of the two treatments was calculated as:

$$SIM = \frac{\{DD\} + \{UU\} - \{DU\} - \{UD\}}{\{DD\} + \{UU\} + \{DU\} + \{UD\} + \{DX\} + \{UX\} + \{XD\} + \{XU\}} \times 100\%$$

where {AB} is the number of genes that were A (=up (U), down (D) or not (X)) significantly regulated in DEN-dosed eggs and B (=U/D/X) regulated in NDELA-dosed eggs.

The Pearson product-moment correlation coefficient was computed between the fold changes of genes in eggs dosed with DEN and NDELA with respect to eggs dosed with vehicle only.

Manual search, Database for Annotation, Visualization and Integrated Discovery (DAVID), the Kyoto Encyclopedia of Genes and Genomes (KEGG), and Ingenuity Pathway Analysis (IPA) software were used for functional annotation, gene ontology analysis, and visualization of data. The "core analysis" function in the IPA software was used to categorize and visualize biological functions and gene networks. For each molecular pathway, p-value was calculated on the basis of a right-tailed Fisher exact test. This test measures an overlap between genes significantly expressed in the experiment and predicted regulated gene set in a particular pathway (Krämer et al., 2014). Pathways with p-value $<.05$ were considered significant.

RESULTS

The viability of embryo-fetuses in control and dosed groups was 100%, indicating absence of toxicity.

Out of 26 145 genes present on the array, 463 genes were selected (manually and using IPA and KEGG software) for analysis based on their role in endo- and xenobiotic biotransformation. Expression of those genes in the vehicle control group (DW) were similar to that in the environmental control group, less than 1% of genes involved in encoding of xenobiotic biotrasformation enzymes were deregulated in DW group. In contrast, DEN at 2 mg/egg and NDELA, at 4 mg/egg produced significant changes in gene expression patterns. As illustrated in Figure 2, most xenobiotic genes were expressed in a similar manner in groups dosed with DEN and NDELA. The similarity (SIM) index of the two groups (see Materials and Methods section) was 90.23% (Figure 2). The correlation between replicas was 0.979, indicating high consistency and high experimental quality. DEN up-regulated 66 and down-regulated 95 of metabolic genes, and NDELA up-regulated 75 and down-regulated 100 genes involved in regulation of chemical metabolism. Both compounds shared 64 up-regulated and 93 down-regulated genes.

IPA functional annotation of significantly deregulated metabolic genes, revealed significant ($p = 6.92E\text{-}21$) enrichment of metabolism signaling molecular pathway by either DEN or NDELA exposure (Figure 3, Supplementary Table 1). Analysis also revealed significant enrichment of cytochrome P450 panel in humans, rat, and mouse (Figure 3), indicating similarities between xenobiotic-related genes expressed in chicken fetuses compared with other species. When comparing DEN and NDELA gene expression profiles, DEN more significantly affected oxidative stress and CAR/RXR activation pathways ($p = 9.83E\text{-}05$ and

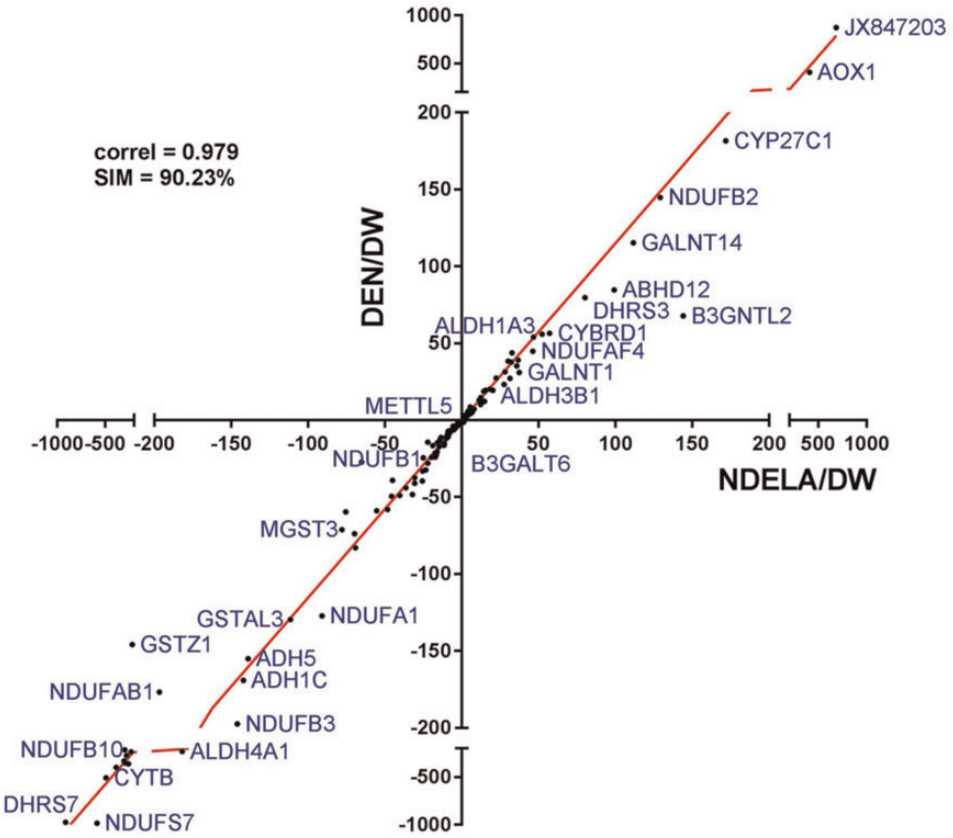

Figure 2. Microarray analysis of gene expression in the chicken fetal livers dosed with diethylnitrosamine (DEN) and *N*-nitrosodiethanolamine (NDELA) with respect to control group dosed with DW. Fold change (negative for down-regulation) of xenobiotic genes in DEN dosed eggs plotted against fold changes in NDELA dosed eggs. Note that most genes fall close to the diagonal (red line) of the equal regulation by the 2 compounds. Gene symbols are shown where space allowed.

9.72E-0.4, respectively) (Figure 3, Supplementary Table 1), which are known to be altered during carcinogenesis.

Tables 2 and 3 present the list of genes which encode phase I and II metabolic enzymes that were significantly modulated by DEN and NDELA. As evident from the tables, chicken fetal liver possesses a wide range of genes involved in xenobiotic transformation. Overall, exposure to DEN and NDELA in chicken fetal livers appeared to affect a higher number of genes regulating phase I xenobiotic metabolism, compared with that of phase II genes. The major difference in gene expression profiles of DEN and NDELA were the expression of cytochrome genes in phase I (Table 2) and genes responsible for glucuronidation in phase II (Table 3). Specifically, for phase I metabolic cytochrome genes responsible for oxidation, *CYB5D2*, *CYP1A5*, and *CYP2AB4*, were up-regulated by NDELA, while DEN did not significantly alter their expression. Additionally, NADPH oxidase gene, *NOX4*, was up-regulated by NDELA only, while abhydrolase gene, *ABHD2*, was up-regulated by DEN only. Over 60% of cytochrome genes detected in chicken livers belonged to either A or B subfamily. DEN and NDELA inhibited more than half of the genes involved in the processes of reduction (68%) and hydrolysis (up to 57%) (Table 2).

Among phase II genes, *B3GALT2*, *B3GNT5*, *B3GNTL1*, *B4GALT5*, *GALE*, *GALNTL4* genes were up-regulated (with exception of *GALE*, which was down-regulated) by NDELA, and not significantly changed in the group that received DEN (Table 3). Additionally, NDELA up-regulated *METTL6*, *NDST1*, *GSTCD* genes, while DEN did not. DEN and NDELA inhibited over 50% of genes responsible for methylation (up to 62%), sulfation (up to 67%), glutathione conjugation (over 79%), and acetylation (100%). In contrast, 60% and 71% of genes involved in glucuronidation, a major detoxication pathway for nitrosamines, were induced by DEN and NDELA, respectively (Table 3).

IPA molecular network analysis also revealed only minor differences between metabolism of DEN and NDELA by embryo-chicken fetal liver (Figs. 4 and 5). The networks altered by DEN and NDELA include genes responsible for oxidation, mainly cytochromes from subfamily B, genes involved in reduction, as well as phase II glutathione conjugation and glucuronidation. Expression of the majority of the genes in the network was inhibited by the nitrosamines (Figs. 4 and 5).

DISCUSSION

In the present study, we have demonstrated that chicken embryo-fetal liver, under the conditions of the CEGA, expresses a wide variety (463) of genes involved in xenobiotic biotransformation. Liver was selected for analyses, since it is the primary organ utilized in CEGA, due to its high metabolic abilities, which in avian fetuses begin to develop on day 5 or even earlier (Clegg, 1964; Hamilton et al., 1983). Such early metabolic activity is due to early differentiation of avian liver, since the development of the avian fetus, in contrast to mammals, is autonomous (Sinclair and Sinclair, 1993). A notable aspect of the study is that influences of many other environmental factors is precluded in CEGA, and thus the effects observed in the model are attributable only to the tested chemicals.

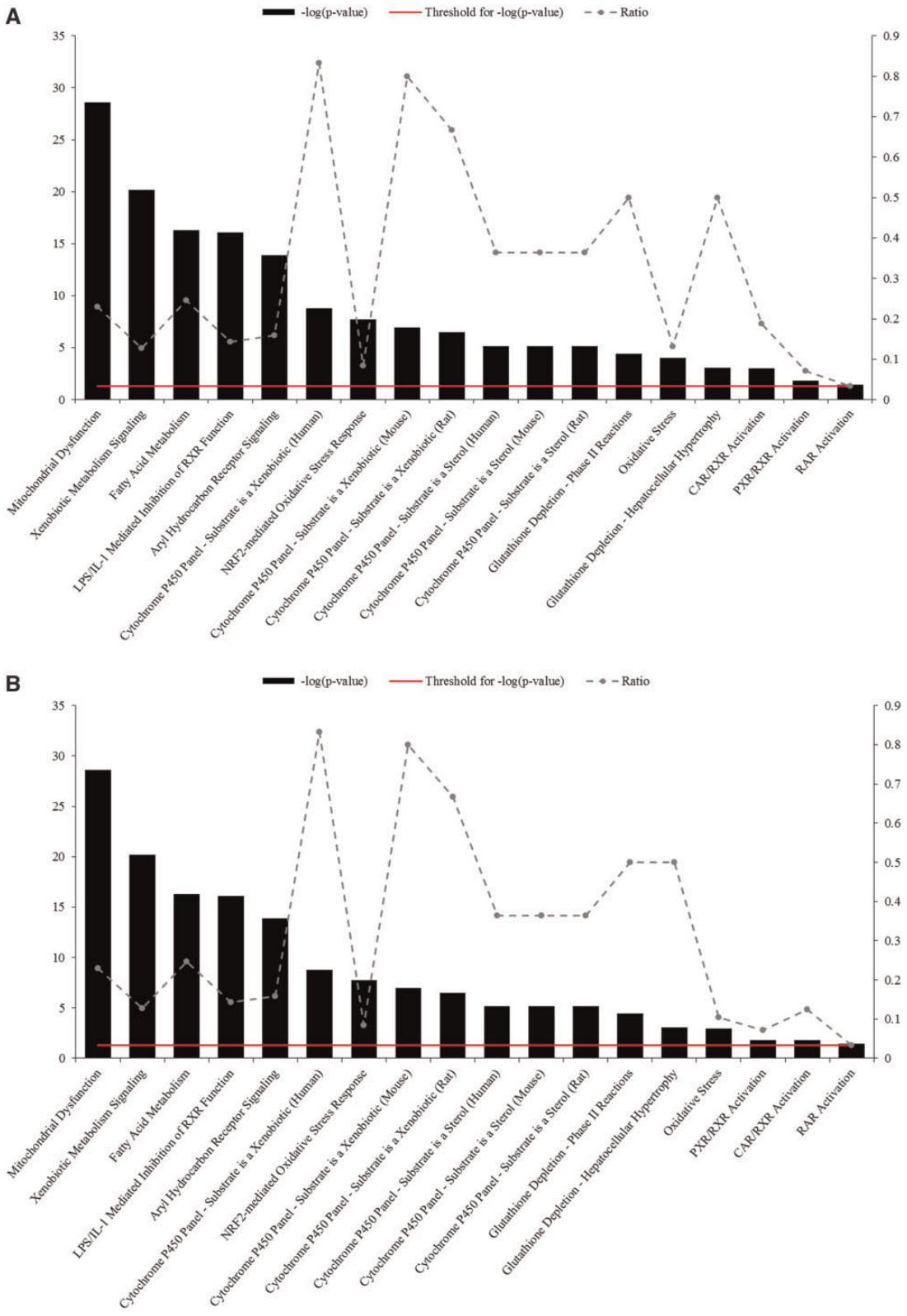

Figure 3. Most significantly impacted pathways in chicken fetal livers exposed to diethylnitrosamine (DEN) (A) and *N*-nitrosodiethanolamine (NDELA) (B). IPA software was used to analyze and visualize pathway enrichment based on the uploaded gene list of significantly deregulated metabolic genes. See Supplementary Table 1 for details.

Table 2. List of Genes Regulating Phase I Enzymes Involved in Endobiotic/Xenobiotic Metabolism in chicken fetal liver Significantly Modulated by Injections With 2 mg/Egg of Diethylnitrosamine (DEN) and 4 mg/egg of *N*-Nitrosodiethanolamine (NDELA)

Symbol	Description	DEN	NDELA
1. Oxidation			
CYTB	Cytochrome b	−510.40	−493.15
CYB5A	Cytochrome b5 type A (microsomal)	−353.72	−294.36
CYB5R2	Cytochrome b5 reductase 2	−82.85	−69.29
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	−49.43	−45.67
CYCS	Cytochrome c, somatic	−48.33	−32.25
CYB5B	Cytochrome b5 outer mitochondrial membrane	−43.95	−36.53
CYP27A1	Cytochrome P450, family 27, subfamily A, polypeptide 1	−39.58	−25.79
CYP2C18	Cytochrome P450, family 2, subfamily C, polypeptide 18	−14.98	−11.50
CYP2AC1	Cytochrome P450, family 2, subfamily AC, polypeptide 1	−14.56	−15.81
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	−11.74	−10.22
CYP3A7	Cytochrome P450 A 37	−11.09	−10.12
CYP1A4	Cytochrome P450 1A4	−3.96	−5.55
CYP4A22	Cytochrome P450 family 4 subfamily A member 22	−3.91	−3.50
CYP4A22	Cytochrome P450 family 4 subfamily A member 2	−3.87	−5.71
CYB5R4	Cytochrome b5 reductase 4	−3.00	−2.67
CYP2U1	Cytochrome P450 family 2 subfamily U member 1	−1.66	−1.34
CYB5D2	Cytochrome b5 domain containing 2	−1.48	−2.41
POR	Cytochrome P450 oxidoreductase	1.67	1.61
CYP2J22	Cytochrome P450, family 2, subfamily J, polypeptide 22	1.70	1.69
CYP1A5	Cytochrome P450	2.05	2.79
CYP20A1	Cytochrome P450, family 20, subfamily A, polypeptide 1	3.68	2.87
CYP2W1	Cytochrome P450 family 2 subfamily W member 1	3.88	4.79
CYP2AB4	Cytochrome P450, family 2, subfamily AB, polypeptide 4	4.24	4.12
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	4.98	4.08
CYBASC3	Cytochrome b, ascorbate dependent 3	5.51	3.95
CYP2C23A	Cytochrome P450, family 2, subfamily C, polypeptide 23a	12.92	11.32
CYP7B1	Cytochrome P450, family 7, subfamily B, polypeptide 1	18.63	14.31
CYP1B1	Cytochrome P450 family 1 subfamily B member 1	27.35	22.37
CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2	55.93	52.22
CYBRD1	Cytochrome b reductase 1	56.57	57.05
CYP27C1	Cytochrome P450, family 27, subfamily C, polypeptide 1	181.57	171.64
CYP2J24P	Cytochrome P450, family 2, subfamily J, polypeptide 24, pseudogene	875.17	684.93
1.1. Non-microsomal oxidation			
ALDH9A1	aldehyde dehydrogenase 9 family member A1	−275.97	−277.75
ALDH4A1	Aldehyde dehydrogenase 4 family, member A1	−234.74	−181.99
ADH1C	Alcohol dehydrogenase 1C (class I), gamma polypeptide	−169.11	−142.19
ADH5	Alcohol dehydrogenase 5 (class III), chi polypeptide	−155.12	−139.00
ALDH3A2	Aldehyde dehydrogenase 3 family, member A2	−73.81	−69.88
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	−13.82	−14.69
ADH6	Alcohol dehydrogenase 6 (class V)	−9.13	−9.36
ALDH8A1	Aldehyde dehydrogenase 8 family, member A1	−5.61	−5.55
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2	2.54	2.72
MAOB	Monoamine oxidase B	13.02	12.42
ALDH3B1	Aldehyde dehydrogenase	20.23	18.20
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	43.74	32.59
AOX1	Aldehyde oxidase 1	408.11	406.60
2. Reduction			
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7	−982.96	−588.23
DHRS7	Dehydrogenase/reductase (SDR family) member 7	−973.54	−914.21
NDUFC2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2	−401.02	−387.56
NDUFB6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	−364.17	−260.10
NDUFA8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	−333.71	−304.55
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	−239.45	−231.93
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	−218.17	−297.59
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3	−197.45	−146.09
NDUFAB1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	−176.69	−196.89
NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	−127.18	−90.95
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5	−58.90	−55.42
NDUFB9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9	−57.96	−48.39
AKR1B1L	Aldo-keto reductase family 1 member B1-like	−24.11	−19.85

Table 2. (continued)

Symbol	Description	DEN	NDELA
DHRS13	Dehydrogenase/reductase (SDR family) member 13	−22.58	−16.75
NQO1	NAD(P)H dehydrogenase, quinone 1	−21.30	−17.48
DHRS7B	Dehydrogenase/reductase (SDR family) member 7B	−17.16	−14.88
NDUFB1	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 1	−16.49	−18.97
C2ORF56	NADH dehydrogenase (ubiquinone) complex I, assembly factor 7	−16.30	−11.68
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9	−14.33	−22.15
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3	−12.14	−13.95
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8	−11.19	−13.53
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8	−10.82	−8.97
NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5	−7.04	−8.13
AKR1B1L	Aldo-keto reductase family 1, member B1-like (aldose reductase)	−6.67	−7.04
NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2	−4.97	−4.81
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10	−3.92	−3.44
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1	−2.90	−2.41
AKR1B10L1	Aldo-keto reductase family 1 member B10-like 1	−2.85	−3.28
NQO2	NAD(P)H dehydrogenase, quinone 2	−2.37	−2.55
DHRS4	Dehydrogenase/reductase (SDR family) member 4	−2.37	−2.13
NOX4	NADPH oxidase 4 (NOX4)	2.12	2.53
NSDHL	NAD(P)-dependent steroid dehydrogenase-like	2.62	2.36
AKR1A1	Aldo-keto reductase family 1, member A1 (aldehyde reductase)	3.41	2.86
NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1	6.22	5.07
NDUFAF1	NADH dehydrogenase (ubiquinone) complex I, assembly factor 1	7.14	7.26
DHRS12	Dehydrogenase/reductase (SDR family) member 12	7.27	7.92
DHRS11	Dehydrogenase/reductase (SDR family) member 11	8.59	5.37
NDUFV3	NADH dehydrogenase (ubiquinone) flavoprotein 3	12.19	11.68
NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2	19.40	15.46
AKR1B10	Aldo-keto reductase family 1, member B10 (aldose reductase)	27.17	31.24
NDUFAF4	NADH dehydrogenase (ubiquinone) complex I, assembly factor 4	44.84	46.20
DHRS3	Dehydrogenase/reductase (SDR family) member 3	79.64	80.15
NDUFAF2	NADH: ubiquinone oxidoreductase complex assembly factor 2	81.60	70.59
NDUFB2	NADH: ubiquinone oxidoreductase subunit B2	144.81	129.12
3. Hydrolysis			
TC382723	Gallus, complete	−39.13	−45.18
EPHX1L	Epoxide hydrolase 1-like	−27.91	−22.21
ABHD13	Abhydrolase domain containing 13	−24.36	−25.14
ABHD5	Abhydrolase domain containing 5	−6.42	−6.43
ABHD11	Abhydrolase domain containing 11	N/A	−7.48
ALPL	Alkaline phosphatase, liver/bone/kidney	−4.38	−4.37
ABHD3	Abhydrolase domain containing 3	N/A	−2.23
EPHX2	Epoxide hydrolase 2, cytoplasmic	−1.57	−1.56
ABHD2	Abhydrolase domain containing 2	1.42	1.18
ABHD17B	Family with sequence similarity 108, member B1	1.74	1.53
ALPP	Alkaline phosphatase	2.85	2.35
ABHD6	Abhydrolase domain containing 6	35.28	35.76
ABHD10	Abhydrolase domain containing 10	37.78	32.21
ABHD12	Abhydrolase domain containing 12	84.66	99.15

Red color indicates significantly ($p \leq .05$) up-regulated genes, green color indicates significantly ($p \leq .05$) down-regulated genes, yellow color indicates genes that did not significantly change their expression. Numbers in columns DEN and NDELA represent the fold change (negative for down-regulation). N/A, not quantified in all four replicas of the condition.

The expression of genes encoding for enzymes involved in phase I and II chemical biotransformation was affected by substrates for the enzymes, two dialkylnitrosamines, DEN and NDELA. These observations are consistent with previous reports of activity of metabolic enzymes in avian liver (Hamilton et al., 1983; Ignarro and Shideman, 1968; Jackson et al., 1986; Perrone et al., 2004; Rifkind et al., 1979, 1994; Sinclair and Sinclair, 1993; Wolf and Luepke, 1997), and the reports that their activity is modulated by the enzyme inducer phenobarbital and other xenobiotics, eg, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene, and ethanol (Perrone et al., 2004; Rifkind et al., 1979, 1994; Sinclair et al., 1989; Sinclair and Sinclair, 1993). Our findings confirm and extend knowledge on the metabolic competency of avian embryo-fetal liver.

Previous testing of DEN and NDELA in CEGA (Table 1) revealed that chicken fetal liver was capable of bioactivation, evidenced by DNA damage and teratogenic effects produced by DEN in the assay, as well as to differentiate genotoxic and non-genotoxic chemicals, despite their structural similarities (Williams et al., 2014). The analysis of gene expression modulations induced by the two nitrosamines contributes to clarification of the mode of action of these chemicals in CEGA. The tested total dose for each compound was the highest dose previously tested in CEGA, which was known for DEN, to produce

Table 3. List of Genes Regulating Phase II Enzymes Involved in Endobiotic/Xenobiotic Metabolism in chicken fetal liver Significantly Modulated by Injections With 2 mg/Egg of Diethylnitrosamine (DEN) and 4 mg/Egg *N*-Nitrosodiethanolamine (NDELA)

Symbol	Description	DEN	NDELA
1. Methylation			
METTL7A	Methyltransferase like 7A	N/A	−125.47
METTL21D	Methyltransferase valosin containing protein lysine (K)	−66.97	−50.59
METTL5	Methyltransferase like 5	−8.38	−8.11
METTL10	Methyltransferase like 10	−8.34	−6.79
METTL9	Methyltransferase like 9	−4.62	−4.01
METTL21A	Methyltransferase like 21A	−4.09	−4.42
METTL11A	Methyltransferase like 11A	−4.02	−3.26
METTL15	Methyltransferase like 15	−2.96	−2.47
METTL6	Methyltransferase like 6	1.11	1.52
METTL2A	Methyltransferase like 2A	3.19	2.82
METTL18	Methyltransferase like 18	4.74	6.11
METTL13	Methyltransferase like 13	19.39	20.18
METTL22	Methyltransferase-like protein 22	23.21	27.48
2. Sulfation			
SULT1B1	Sulfotransferase family, cytosolic, 1B, member 1	−59.58	−75.50
SULT1E1	Sulfotransferase family 1E, estrogen-preferring, member 1	−33.02	−25.56
SULT	Sulfotransferase	−8.43	−8.91
NDST2	*N*-Deacetylase and *N*-sulfotransferase 2	−2.45	−2.33
SULT6B1L	Sulfotransferase family, cytosolic, 6B, member 1-like	−1.62	−1.31
SULT1C3	Sulfotransferase family, cytosolic, 1C, member 3	N/A	−459.77
NDST1	*N*-Deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 1	1.39	2.05
SULT6B1	Sulfotransferase family, cytosolic, 6B, member 1	12.30	14.69
NDST3	*N*-Deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 3	31.45	28.11
3. Conjugation with glutathione			
GPX1	Glutathione peroxidase 1	−1147.61	N/A
GSTZ1	Glutathione S-transferase zeta 1	−145.76	−219.59
GSTAL3	Glutathione S-transferase class-alpha-like 3	−129.59	−111.58
MGST3	MICROSOMAL glutathione S-transferase 3	−71.22	−78.12
GPX4	Phospholipid hydroperoxide glutathione peroxidase	−69.71	−96.71
GSTM2	Glutathione S-transferase mu 2 (muscle)	−48.89	−40.49
GSR	Glutathione reductase	−40.95	−30.70
GSTA	Glutathione S-transferase class-alpha	−27.26	−65.28
GSTO1	Glutathione S-transferase omega 1	−23.77	−17.78
GSTA3	Glutathione S-transferase alpha 3	−6.26	−5.31
GPX3	Glutathione peroxidase 3	−2.81	−2.01
GSTCD	Glutathione S-transferase, C-terminal domain containing	1.22	1.65
MGST2	Microsomal glutathione S-transferase 2	4.60	5.85
GSS	Glutathione synthetase	5.89	4.63
4. Acetylation			
NAT8B	*N*-Acetyltransferase 8B (GCN5-related, putative, gene/pseudogene)	−20.66	−16.13
NAT	*N*-Acetyltransferase, liver isozyme	−11.58	−9.91
NAT8L	*N*-Acetyltransferase 8-like (GCN5-related, putative)	−5.00	−4.24
NAT9	*N*-Acetyltransferase 9 (GCN5-related, putative)	−4.25	−4.74
5. Glucuronidation			
GALNT7	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 7	−37.27	−30.72
UGDH	UDP-glucose 6-dehydrogenase (UGDH)	−32.39	−23.46
B4GALT2	UDP-Gal: betaGlcNAc beta 1, 4-galactosyltransferase, polypeptide 2	−23.97	−17.33
UGP2	UDP-glucose pyrophosphorylase 2 (UGP2)	−7.22	−8.96
B4GALT4	UDP-Gal: betaGlcNAc beta 1, 4-galactosyltransferase, polypeptide 4	−6.93	−8.29
UXS1	UDP-glucuronate decarboxylase 1	−5.99	−5.15
B4GALT7	Xylosylprotein beta 1, 4-galactosyltransferase, polypeptide 7 (galactosyltransferase I)	−5.74	−6.21
B3GNT2	UDP-GlcNAc: betaGal beta-1, 3-N-acetylglucosaminyltransferase 2	−4.74	−4.76
GALE	UDP-galactose-4-epimerase	−1.24	−1.58
B3GNTL1	UDP-GlcNAc: betaGal beta-1, 3-*N*-acetylglucosaminyltransferase-like 1	1.47	1.57
B3GALT2	UDP-Gal: betaGlcNAc beta 1, 3-galactosyltransferase, polypeptide 2	1.50	1.80
B4GALT5	UDP-Gal: betaGlcNAc beta 1, 4-galactosyltransferase, polypeptide 5	1.56	1.94
GALNTL4	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-like 4	1.79	2.23
UGGT1	UDP-glucose glycoprotein glucosyltransferase 1	2.34	1.78
UGT2A3	UDP glucuronosyltransferase 2 family, polypeptide A1	2.47	3.25
B4GALT1	UDP-Gal: betaGlcNAc beta 1, 4-galactosyltransferase, polypeptide 1	2.75	2.72

Table 3. (continued)

Symbol	Description	DEN	NDELA
B3GNT5	UDP-GlcNAc: betaGal beta-1, 3-*N*-acetylglucosaminyltransferase 5	3.19	3.12
UGT8	UDP-galactose ceramide galactosyltransferase	3.20	2.94
UGCG	UDP-glucose ceramide glucosyltransferase	3.28	3.22
GALNT11	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 11	3.41	2.37
B3GNT7	UDP-GlcNAc: betaGal beta-1, 3-*N*-acetylglucosaminyltransferase 7	4.30	4.49
B3GAT1	beta-1, 3-glucuronyltransferase 1 (glucuronosyltransferase P)	4.80	2.61
GALNT6	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 6	5.38	6.71
GALNT12	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 12	5.83	4.02
UGGT2	UDP-glucose glycoprotein glucosyltransferase 2	6.38	6.07
GALNT10	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 10	7.23	6.18
B3GALT6	UDP-Gal: betaGal beta 1, 3-galactosyltransferase polypeptide 6	10.21	12.24
B3GALTL	beta 1, 3-galactosyltransferase-like	10.41	9.65
GALNTL1	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 16	14.74	12.28
GALNT1	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 1	31.02	37.33
GALNTL6	polypeptide *N*-acetylgalactosaminyltransferase-like 6	38.41	30.19
GALNT13	UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 13	39.06	36.56
B3GNT9	UDP-GlcNAc: betaGal beta-1, 3-N-acetylglucosaminyltransferase 9	53.98	46.67
B3GNTL2	UDP-GlcNAc: betaGal beta-1, 3-N-acetylglucosaminyltransferase-like 2	67.86	143.99
GALNT14	UDP-N-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 14	115.30	111.64

Red color indicates significantly ($p \leq .05$) up-regulated genes, green color indicates significantly ($p \leq .05$) down-regulated genes, yellow color indicates genes that did not significantly change their expression. Numbers in columns DEN and NDELA represent the fold change (negative for down-regulation). N/A, not quantified in all four replicas of the condition.

effects on both molecular and phenotypic levels, in contrast to NDELA, but not to significantly affect viability of fetuses (Iatropoulos et al., 2017; Williams et al., 2014). Our analyses allows for phenotypic anchoring of chemical-induced gene expression changes with genotoxicity and morphological responses.

Mapping of selected metabolic genes onto IPA-established pathways of xenobiotic metabolism regulation (Supplementary Figure 1) revealed a number of genes present in chicken embryo-fetal livers with orthologs in other organisms. DEN and NDELA exposure in CEGA enriched several pathways consistent with binding and activation of several nuclear receptors: aryl hydrocarbon receptor (AHR), constitutive androstane receptor (CAR), and pregnane X receptor (RXR) (Supplementary Figure 1).

The effects of DEN on gene expression levels overlapped with that of NDELA for the majority of selected genes (Figure 2), which would be expected, since the compounds possess similarities in chemical structures as well as in pathways of biotransformation (Figure 1). Nevertheless, some differences were present, especially in the expression of genes responsible for oxidation and glucuronidation of nitrosamines. This might partially explain the difference in the genotoxic potential of DEN and NDELA in CEGA.

For many xenobiotics, including nitrosamines, biotransformation to a reactive moiety results from oxidation reactions catalyzed by cytochromes. In avian species, many forms of cytochrome P450 are well characterized (Lorr and Bloom, 1987; Sinclair and Sinclair, 1993). The induction of mixed-function oxidase system in avian embryo-fetus has been recorded as early as 3 days of development, with levels of activity being comparable with those reported for adult chicken and other species and thus, considered sufficient for metabolic activation of procarcinogens (Hamilton et al., 1983). Moreover, the activity of mixed function oxidases was reported not to depend significantly on the sex of the chicken (Rifkind et al., 1979). Consistently with other species, chicken possesses two genes that belong to CYP1A subfamily (*CYP1A4* and *CYP1A5*) homologous to mammalian *CYP1A1* and *CYP1A2*, which were reported to have overlapping but distinctly different functions (Gilday et al., 1996; Goldstone and Stegeman, 2006; Yang et al., 2013). Other isoforms in avian CYP1-3 families were also identified (Watanabe et al., 2013). Consistent with reports in the literature, exposure to DEN and NDELA significantly up-regulated expression of *CYP2C23a* which is induced by chicken xenobiotic receptor (CXR) activator, phenobarbital (Watanabe et al., 2013). High expression levels of *CYP2J24P*, *CYP27C1*, *CYBRD1*, and *CYP21A2* (Table 2) indicate their importance in metabolism of nitrosamines in chickens. In humans only one *CYP2J* is present, while in avian species multiple genes of this subfamily were identified (Watanabe et al., 2013). DEN and NDELA strongly down-regulated the expression of cytochromes from B subfamily, ie, *CYTB*, *CYB5A*, and *CYB5R2*, genes also involved in endobiotic metabolism. Such strong inhibition of gene expression could be associated with the depletion of enzymes due to high doses of DEN and NDELA tested, or possibly these enzymes are not utilized in the metabolism of nitrosamines by chicken fetal liver.

Genes from CYP2C subfamily were also modified by DEN and NDELA exposure in CEGA (Table 2). These genes, formerly called *CYP2H* (Watanabe et al., 2013), encode for enzyme highly inducible by phenobarbital and other xenobiotics (Sinclair et al., 1990).

DEN did not significantly modify the expression of 3 cytochrome genes: *CYB5D2*, *CYP1A5*, *as CYP2AB4* (gene found only in avian species) (Table 2). It is possible that this difference contributes to a difference in metabolic activation of DEN and NDELA by chicken fetal liver. It is also possible that since these genes are not regulated by DEN, the activity of cytochromes, which are involved in detoxication of DEN by denitrosation, is inhibited.

Published data suggest the importance of CYP2E1 in biotransformation of NDELA via α-hydroxylation pathway, producing an α-hydroxy metabolite which exhibits high levels of

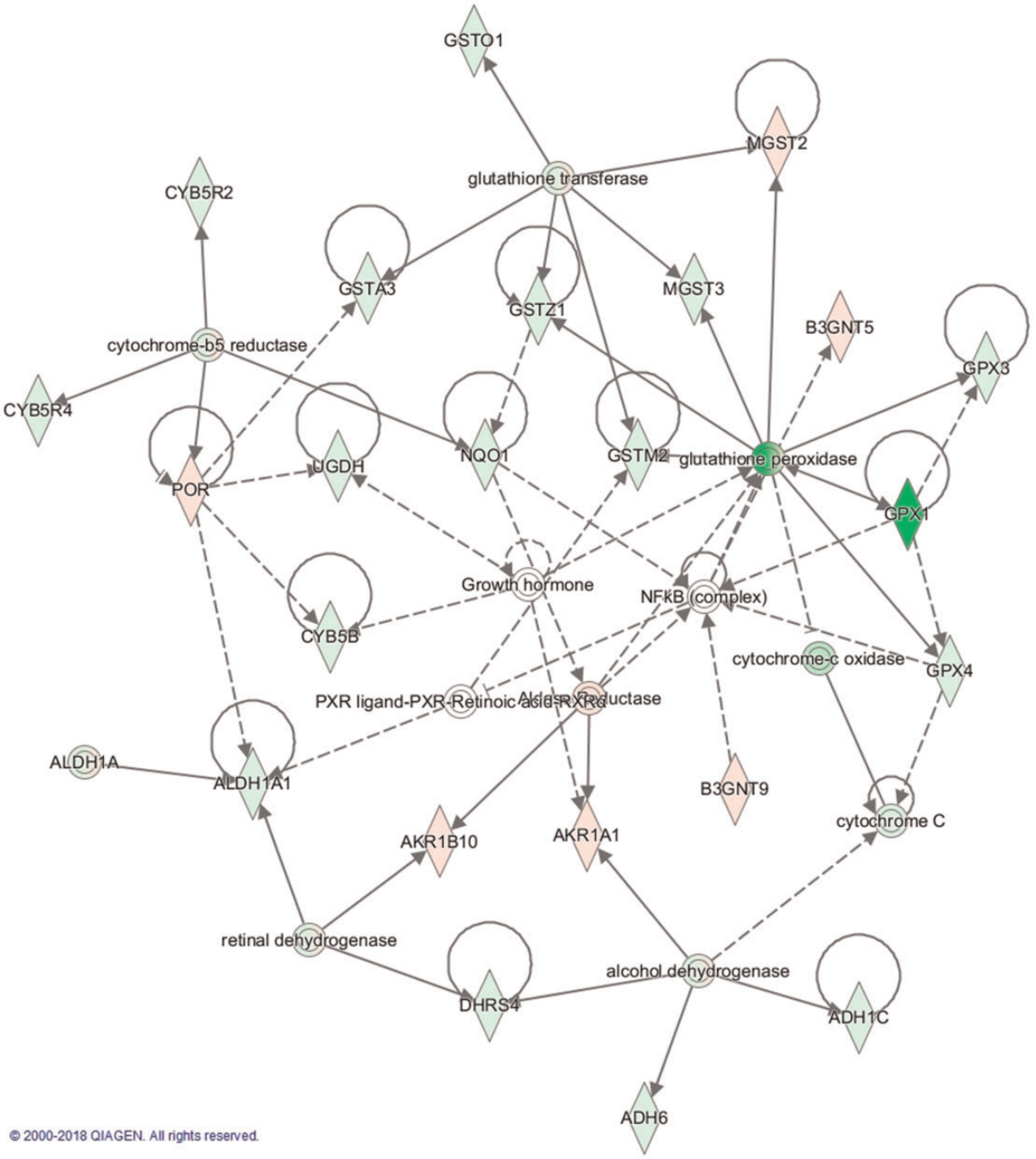

Figure 4. Molecular network interactions of metabolic genes regulated by diethylnitrosamine (DEN) in chicken fetal liver. The IPA database was used to determine and visualize molecular pathways enrichment by the significantly deregulated metabolic genes. Red indicates up-regulated genes. Green indicates down-regulated genes. Note that most genes in the network are inhibited by 2 mg/egg of DEN.

cytotoxicity (IARC, 2000; Loeppky, 1999). The avian liver does not possess *CYP2E* genes (Watanabe et al., 2013), which possibly results in the lack of toxicity of NDELA in CEGA (Iatropoulos et al., 2017; Williams et al., 2014). This correlates with absence of genotoxicity of NDELA *in vivo*, due to predominant metabolism via β-oxidation (Lijinsky, 1987).

Avian and mammalian sulfotransferases (SULT) have been determined to be closely related structurally and functionally (Wilson et al., 2004). In a study conducted by Sterzel and Eisenbrand (1986), inhibition of SULT was shown to abolish DNA single strand breaks produced by NDELA in rat liver. In the current study, the majority of genes controlling SULT activity were inhibited by DEN and NDELA. Consistent with the findings of Sterzel and Eisenbrand in rats, NDELA did not produce DNA strand breaks in chicken fetal livers (Williams et al., 2014), which could be related to inhibition of SULTs. DEN did produce DNA strand break in CEGA, suggesting that SULT inhibition might not be sufficient to abolish its genotoxicity.

Genes encoding for ALD, another important enzyme in metabolism of nitrosamines (Loeppky, 1999) (Figure 1), were

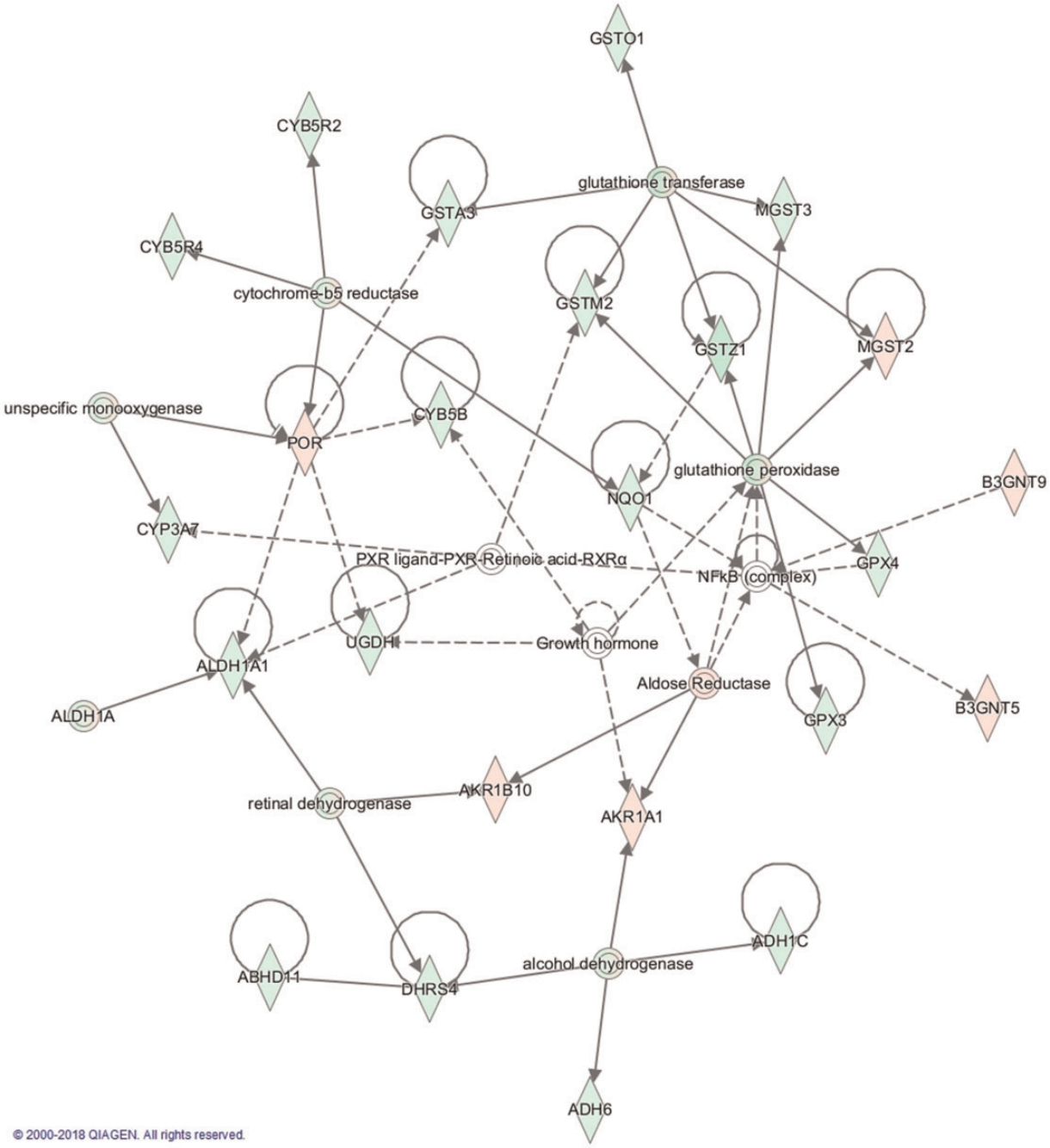

Figure 5. Molecular network interactions of metabolic genes regulated by *N*-nitrosodiethanolamine (NDELA) in chicken fetal liver. The IPA database was used to determine and visualize molecular pathways enrichment by the significantly deregulated metabolic genes. Red indicates up-regulated genes. Green indicates down-regulated genes. Note that most genes in the network are inhibited by 4 mg/egg of NDELA.

down-regulated by exposure to DEN and NDELA (Table 2), which could again indicate their depletion by high doses of xenobiotics. Meanwhile several aldehyde dehydrogenase (ALDH) genes from family 1 and aldehyde oxidase genes, *AOX1*, *ALDH1A3*, *ALDH1B1*, and *ALDH1A2* were significantly induced by exposure to DEN and NDELA. Previous reports also describe the increase of ALDH in rats with liver tumors produced by DEN and described that over half of DEN-produced hepatocellular carcinomas expressed tumor-associated ALDH phenotype (Canuto et al., 1993; Lindahl and Evces, 1987; Wischusen et al., 1983). While changes in ALDH activity in rodents are expressed in late stages of hepatocarcinogenesis, changes in CEGA were observed within 3 days of dosing. Thus, it is possible that induction of ALDH contributes to genotoxicity exhibited by DEN in CEGA. However, no major differences in the expression of ALDH genes were noticed between DEN- and NDELA-dosed eggs making it difficult to account for the role of these genes in genotoxicity of DEN.

The major difference between activity of xenobiotic-related genes in chicken fetal livers after dosing with DEN or NDELA were found in the group of genes that code for UDP glucuronyl transferase (UGT) activity (Table 3). It was reported that

nitrosamines are conjugated in rat hepatocytes by UDP UGT, and the extent of glucuronidation depends on the lipophilicity of these chemicals (Wiench et al. 1992). Glucuronidation conjugates of nitrosamines are excreted in urine in laboratory animals and humans and are believed to be detoxication products (Hecht, 1997). In the current study, over 70% of genes encoding for UGT activity were up-regulated by NDELA, which possibly enhanced its detoxication and elimination, confirmed by negative results for its genotoxicity testing in CEGA previously. Meanwhile, DEN-dosed groups had fewer up-regulated glucuronidation genes (Table 3), which probably contributes to its genotoxicity due to lower rates of conjugation and elimination of the compound compared with NDELA.

While glutathione S-transferase (GST) levels were shown to increase in the livers of rats in response to DEN exposure (Canuto et al., 1993; Marinho et al., 1997), and preneoplastic foci produced by DEN in rat liver are positive for placental GST (Hosokawa et al., 1989; Satoh and Hatayama, 2002). In contrast, in CEGA the expression of genes coding for GST activities were mostly down-regulated (Table 3), possibly due to saturation of the pathway by the high dose of nitrosamines. Marked decrease of glutathione transferase activities was previously described after exposure to peroxisome proliferators, eg, nafenopin, clofibrate, due to binding to the enzyme subunit (Furukawa et al., 1985).

The pathway analysis in IPA revealed a network of genes that encode for metabolic enzymes that are known to play an important role in oxidation/hydroxylation of DEN and NDELA *in vivo*, specifically cytochromes, and ALD (Figs. 1, 4, and 5), as well as enzymes crucial for detoxication of nitrosamines, GST, and UGT. This further confirms similarities between xenobiotic metabolism *in ovo* and *in vivo*.

In summary, gene expression profiling in chicken fetal liver confirmed that this organ has an extensive metabolic capacity, which mimics *in vivo* systems. The majority of genes were deregulated in a similar fashion by DEN and NDELA, indicating similarity in the metabolism of *N*-nitrosamines in CEGA. Difference in expression of cytochrome and glucuronidation genes could contribute to differences in the effects of DEN and NDELA in CEGA. Thus, the reported sensitivity of the CEGA to a wide variety of genotoxic carcinogens known to require bioactivation is supported by the documented expression of genes for the enzymes involved. The findings strengthen the hypothesis that *in ovo* models are attractive alternatives to assess a variety of critical endpoints of chemical carcinogenesis.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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