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Comparative evaluation of phenobarbital-induced CYP3A and CYP2H1 gene expression by quantitative RT-PCR in Bantam, Bantamized White Leghorn and White Leghorn chicks

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The present work was to study induction of cytochrome P450 (CYP)3A and CYP2H1 gene by reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR in Bantam, Bantamized White Leghorn and White Leghorn chicks. Out of 18 chicks total 3 from each group (Bantam, Bantamized White Leghorn and White Leghorn) were treated intraperitoneal with phenobarbital at the dose rate of 12 mg/100 g (body weight) while the control group was treated with the saline. Total RNA was extracted from the liver samples using Tri Reagent based method. First strand cDNA was synthesized using one step RT-PCR kit. The PCR was performed and the product was subjected to agarose gel electrophoresis. Quantitative RT-PCR was conducted to quantify gene expression level of CYP3A and CYP2H1 genes. Relative expression ratio of CYP3A and CYP2H1 genes was calculated using relative expression software tool (REST). It was found that CYP3A is up regulated by factor of 1.34, 14.51 and 1.00 in Bantam, Bantamized White Leghorn and White Leghorn chicks, respectively. In Bantam and Bantamized White Leghorn chicks CYP2H1 gene was up regulated by factor 1.50 and 80.87, respectively but down regulated by a factor of 1.97 in White Leghorn chicks. The PCR efficiency ranged from 1.30 to 1.70, 0.86 to 1.70 and 0.91 to 1.58 for CYP3A, CYP2H1 and β-actin, respectively in Bantam, Bantamized White Leghorn and White Leghorn chicks.

Key words: cytochrome P450, CYP3A, CYP2H1, phenobarbital, RT-PCR

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Introduction

The superfamily of cytochrome P450 (CYP) comprises about 500 microsomal, mitochondrial and bacterial proteins that are involved in the metabolism of endogenous and exogenous compounds [15]. Cytochrome families 1, 2, and 3 play an essential role in the biotransformation of xenobiotics, which is critical in the first step of detoxication. The term induction denotes a dose-dependent increase in monooxygenase activity associated with an increase in the level of CYP protein. Induction of CYP-dependent activities enhances the metabolic activation of substrates, thus indirectly determining their toxic, mutagenic and pharmacologic effects. The level of induction and the clinical outcome varies depends on genetic, dietary, physiological and environmental factors. CYP induction response influence CYP-dependent drug metabolism, pharmacokinetics, and drug-drug interactions, the toxicity and carcinogenicity of foreign chemicals, and the activity and disposition of endogenous hormones [3].

Five different classes of prototypical inducer-drugs that activates distinct but overlapping classes of CYPs have been confirmed. One of them is phenobarbital (PB), which is a prototype of a large group of structurally unrelated chemicals that induce a large subset of CYP genes like CYP2A, CYP2B, CYP2C, CYP2H, CYP3A, CYP6A, and CYP102/106. In addition to CYPs, PB induces a large number of other enzymes such as NADPH CYP reductase and specific transferases, increasing metabolic capabilities [19]. These CYPs, as well as more than 50 other genes, are affected by PB and a number of structurally unrelated compounds classified as PB-like inducers [3]. PB induction is observed from a wide variety of species ranging from *Bacillus megaterium* to human [4]. In vertebrates, drug induction occurs predominantly in the liver, intestine while lesser extent extrahepatic tissues, such as skin, kidney, lung, and brain [2].

CYP3As are the predominant P450s expressed in mammalian

liver. CYP3A4 catalyzes the metabolism of 40 to 60 % of all clinically used drugs. Four major classes of CYP3A inducers have been defined. They are steroids, including glucocorticoids and antiglucocorticoids (e.g. RU-486), barbiturates (e.g., PB), macrolide antibiotics (e.g., rifampicin), and antifungal agents (e.g., azoles and imidazoles).

Although CYP3A enzymes are well described in many mammalian species, little is known about these proteins in chicken. The present study was to compare induction and quantification of CYP3A and CYP2H1 gene by PB in Bantam, Bantamized White Leghorn and White Leghorn breeds of chicks.

Materials and Methods

Chemicals and Instruments

Chemicals and instruments for this study were as follows; TRI reagent (Sigma Aldrich, USA), reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Qiagen, India), EDTA (Sigma Aldrich, USA), MOPS (Sigma Aldrich, USA), agarose (Sigma Aldrich, USA), RNA later (Sigma Aldrich, USA), PB (Smarth Pharma, India), 2X PCR Master mix (Genetix, India), ethidium bromide (Sigma Aldrich, USA), Mastercycler gradient (Eppendorf, Germany), gel documentation systems (Gene Genius Bio Imaging; Syngene, UK) and spectro-photometer (Unicam, UK).

Experimental animal

One week old chicks of Bantam, Bantamized White Leghorn and White Leghorn breeds were used in the present study. Out of 18 chicks of Bantam, Bantamized White Leghorn and White Leghorn, 3 from each group were treated intraperitoneal with PB at the dose of 12 mg/100 g (body weight) and control group were treated with same volume of 0.9% normal saline. After 24 hrs, they were sacrificed and liver samples were collected from each bird. The samples were then immediately dipped for few seconds into 2 mM EDTA solution to remove all blood cells. To prevent RNA degradation they were then dipped in 1 ml RNA later solution. The excised samples were then immediately plunged and kept in liquid nitrogen until used.

RNA extraction

Total RNA from tissue samples was extracted using TRI reagent based protocol. RNA was quantified by spectro-photometric analysis using the convention as with 1 absorbance unit at 260 nm equals 40 µg RNA per ml. The U.V. absorbance was checked at 260 and 280 nm for determination of sample concentration and purity. The quality of extracted RNA was assessed by 1% formaldehyde agarose gel electrophoresis using 1% MOPS as gel running buffer.

RT-PCR

First strand cDNAs were synthesized using one step RT-PCR kit with gene specific primers CYP3A (F)-5' GAATAC CGCAAAGGCTTCTTGG 3', CYP3A (R)-5' GAACTCTT CTGGGTTTGGC 3', CYP2H1 (F)-5' GACACTTGACATC TCTTCCTC 3', CYP2H1 (R)-5' CTGGGCATTGACTATC ATT 3', β-actin (F)-5' CCCTGAACCCCAAAGCCAAC 3' and β-actin (R)-5' GACTCCATACCCAAGAAAGA 3' [17]. PCR was conducted in a final reaction volume of 25 ml using 0.2 ml thin wall PCR tube. A master mix for at least 10 samples was prepared and aliquoted in 22 ml quantities in each PCR tube. Three ml sample of cDNA was added in each tube to make the final volume of 25 ml. Thermocycling conditions was set as the followings, initial denaturation at 94°C for 60 sec, denaturation at 94°C for 45 sec, annealing temp 60°C for 45 sec, extension temp 72°C for 90 sec and final extension at 72°C for 300 sec.

To confirm PCR amplification, 5 ml of PCR product mixed with 1 ml of 6X gel loading dye from each tube were electrophoresed on 1% agarose gel (depending on the expected size of amplified product) containing 1% ethidium bromide (5 ml/100 ml) at constant voltage 80 V for 60 min in 0.5X TBE. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

Quantitative RT-PCR

Quantitative RT-PCR was conducted with 1 µg/µl concentration of RNA. First strand cDNA synthesis was carried out using the protocols. PCR was carried out in a final reaction volume of 25 ml using 0.2 ml thin wall PCR tube. A 100 µl master mix for CYP3A was prepared and aliquoted in 25 ml quantities in 4 PCR tubes to carry out sampling at 26th, 31st, 36 th and 41st cycle. Other samples undergo same treatment as well. The above reaction is repeated for CYP2H1 and β-actin primers involving the same samples. Samples were then dropped at 26th, 31st, 36th and 41st cycles. Steps and conditions of thermocycling for PCR is same as previous.

To quantify the gene expression of CYP3A, CYP2H1 and β-actin, the samples were run on 1% agarose gel electrophoresis. 5 ml PCR product of CYP3A, CYP2H1 and β-actin for 26th cycle were mixed in 0.2 ml PCR tubes. To it 1 ml of 6X gel loading dye and 2 ml of gel star was added and incubated for 10 min. The mixture was then loaded in 1% agarose gel containing 1% ethidium bromide solution (5 ml/100 ml) and electrophoresed at constant voltage 80 V for 60 min in 0.5X TBE.

Data analysis

By using GeneTool software (Syngene, UK) quantitation of quantity for CYP3A, CYP2H1 and β-actin with reference to Standard 1 Kb DNA ladder Gene Ruler (Fermentas, Canada) was determined. Relative expression ratio for CYP3A and CYP2H1 gene was calculated by relative expression software tool (REST) [18]. Relative expression ratio of target gene CYP3A was calculated by REST using

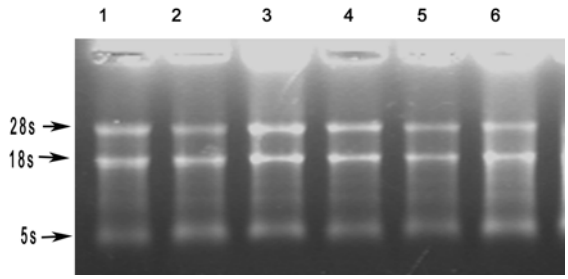

Fig. 1. Formaldehyde agarose gel electrophoresis of RNA sample from phenobarbital treated Bantam chicks.

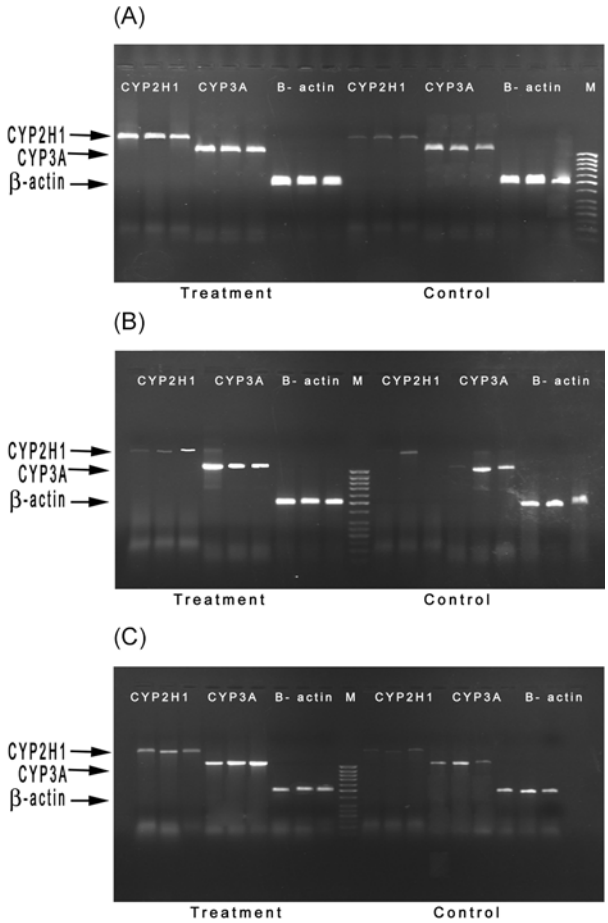

Fig. 2. PCR product run on 1% agarose gel electrophoresis of CYP3A, CYP2H1 and β-actin genes in control and phenobarbital treated (A) Bantam, (B) Bantamized White Leghorn and (C) White Leghorn chicks.

following formula:

$$R = \frac{(E_{target})^{\Delta CP\ target\ (Sample-Control)}}{(E_{ref})^{\Delta CP\ ref\ (Sample-Control)}}$$

In this formula, R represent a relative expression ratio of target gene, E is PCR efficiency, ΔCP is a crossing point difference of an unknown sample versus a control, and ref represent a reference gene. LingPCR software was used to calculate PCR efficiency of CYP3A, CYP2H1 and β-actin genes.

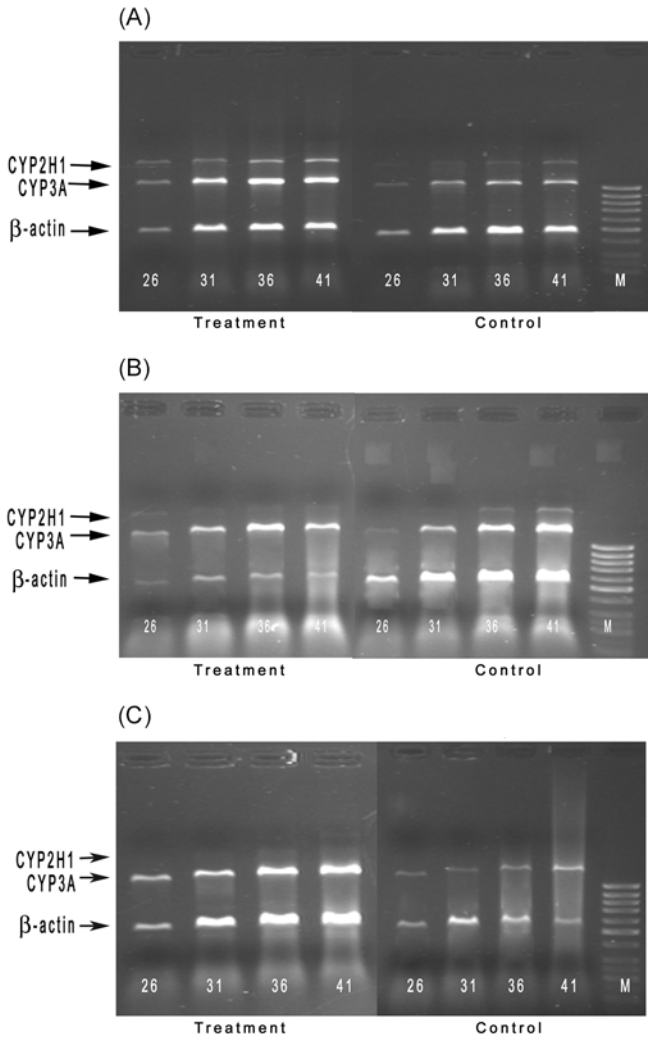

Fig. 3. Quantitative RT-PCR product run on 1% agarose gel electrophoresis for CYP3A, CYP2H1 and β-actin genes in control and phenobarbital treated (A) Bantam, (B) Bantamized White Leghorn and (C) White Leghorn chicks.

Result

All the liver samples processed yielded reasonable quantity (1.8) of RNA. The ratio of absorbance values at 260 and 280 nm was close to 2 indicating low protein and DNA contamination with RNA. All the extracted RNA samples produced compact bands of 28 s, 18 s and 5 s rRNA subunits as shown in Fig. 1.

The PCR was performed at all the samples of Bantam, Bantamized White Leghorn, and White Leghorn chicks using CYP3A, CYP2H1 and β-actin gene specific primers. Specificity of the desired PCR products (cDNA) was ascertained by high resolution agarose gel electrophoresis. It

Table 1. Quantity of CYP3A, CYP2H1 and β-actin gene in phenobarbital treated and control groups of Bantam, Bantamized White Leghorn and White Leghorn chicks (unit: ng/5 µl)

Breed	Gene	Treatment				Control			
	Cycle	26	31	36	41	26	31	36	41
Bantam	CYP 3A	6.74	24.34	28.19	29.81	2.92	9.07	12.90	14.37
	CYP2H1	3.56	5.53	7.93	8.58	0.74	1.08	3.07	3.38
	β-actin	6.85	28.32	30.01	30.87	9.95	28.24	34.20	31.72
Bantamized White Leghorn	CYP 3A	3.99	7.85	12.85	9.26	2.19	6.33	12.41	14.34
	CYP2H1	1.08	0.80	1.11	0.59	-	-	1.67	2.72
	β-actin	2.40	5.57	5.32	4.68	9.95	16.25	21.94	21.00
White Leghorn	CYP 3A	15.04	20.04	30.33	32.86	3.25	2.95	5.09	10.73
	CYP2H1	-	-	0.12	0.41	-	-	-	-
	β-actin	13.13	31.18	40.70	38.87	7.13	18.41	13.32	7.52

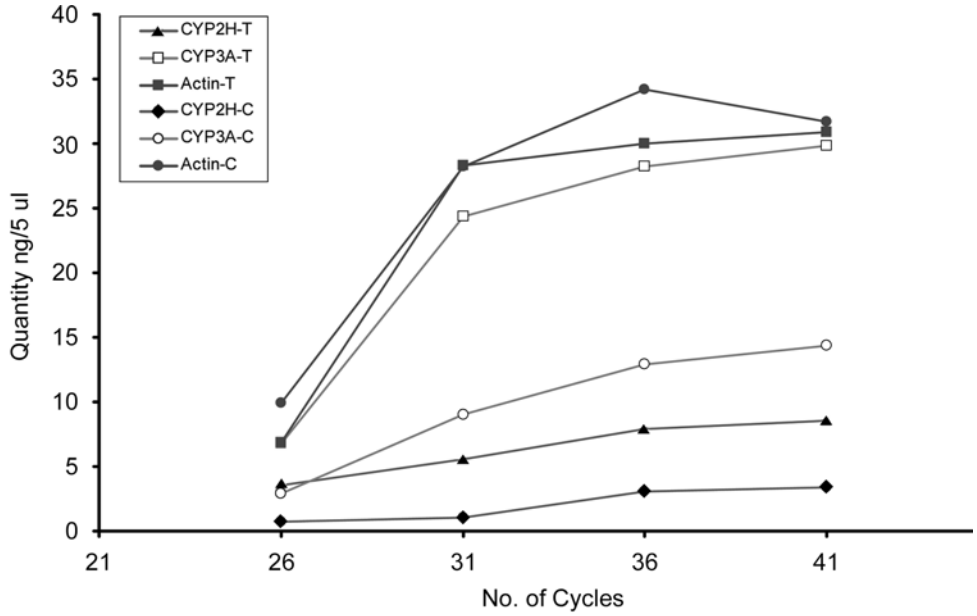

Fig. 4. Quantity of CYP2H1, CYP3A and β-actin genes in phenobarbital treated and control Bantam chicks at 26, 31, 36 and 41 cycles of quantitative PCR (T-treatment C-control).

revealed an amplicon of 1107 bp, 1576 and 486 bp for CYP3A, CYP2H1 and β-actin genes, respectively in all three breeds of chicks as shown in Fig. 2.

Quantitative RT-PCR was undertaken to quantify expression level of CYP3A, CYP2H1 and β-actin genes. PCR amplifications were sampled at 26th, 31st, 36th and 41st cycles. The PCR products were electrophoresed and the comparison in amplification for the three genes after each cycle was presented in Fig. 3. The quantity of CYP3A, CYP2H1 and β-actin was calculated using Gene Tool software with 1 kb DNA ladder having concentration of 7.1 ng/0.5 µl at 500 bp as reference. Quantity of expressed CYP3A, CYP2H1 and β-actin gene was presented in Table 1. The same figures for representation group of chicks were presented graphically in the Fig. 4. Relative expression ratio of CYP3A, CYP2H1 and β-actin genes was presented in Table 2. PCR efficiency for each sample was calculated using the formula $E = 10^{(-1/slope)}$. For CYP3A, CYP2H1 and β-actin 26th, 31st and 36th cycle points were considered and 41st cycle was omitted as amplification was not exponential from 36th to 41st cycle. The values of PCR efficiency were depicted in Table 3 for CYP3A and CYP2H1 genes. The PCR efficiency in all three breeds of chicks ranged from 1.30 to 1.70, 0.86 to 1.70 and 0.91 to 1.58 for CYP3A, CYP2H1 and β-actin genes, respectively.

Discussion

PB is one of the five prototypical inducer classes. PB and PB- type inducers affect the transcription of the CYP2B, CYP2C and CYP3A subfamily gene predominantly in addition to at least 50 other genes in the liver [3,13,19].

Table 2. Gene expression ratio of CYP 3A and CYP2H1 genes in Bantam, Bantamized White Leghorn and White Leghorn treated with phenobarbital

Breed	CYP3A		CYP2H1	
	Expression ratio	Regulation of gene	Expression ratio	Regulation of gene
Bantam	1.34	Up regulation	1.50	Up regulation
Bantamized White Leghorn	14.51	Up regulation	80.87	Up regulation
White Leghorn	1.00	Up regulation	1.97	Down regulation

Table 3. PCR efficiency of CYP3A, CYP2H1 and β-actin genes in control group and phenobarbital treated Bantam, Bantamized White Leghorn and White Leghorn (WLH) chicks

Group	Gene	Bantam		Bantamized WLH		WLH	
		PCR efficiency	R^2	PCR efficiency	R^2	PCR efficiency	R^2
Phenobarbital treated group	CYP3A	1.575	0.700	1.737	0.842	1.318	0.947
	CYP2H1	1.359	0.945	0.862	0.000	-	-
	β-Actin	1.580	0.646	0.917	0.931	1.761	0.915
Control Group	CYP3A	1.671	0.826	1.347	0.945	1.511	0.820
	CYP2H1	1.751	0.913	-	-	-	-
	β-Actin	1.443	0.671	1.289	0.817	1.367	0.420

Transcriptional induction of CYP by PB has been observed in different species such as mammals, birds and bacteria [13,19]. The inductive response can have a major influence on drug-drug interaction, toxicity and carcinogenicity of exogenous and endogenous compounds [3]. PB treatment increases the transcription on number of CYP genes in chicken (CYP2H1/2, CYP3A and CYP2C45), rat (CYP3A1 /2 and CYP2B1/2), mouse (CYP2b10/9), rabbit (CYP2C1/ 2), dog (CYP 2B11), human (CYP3A) and *B. Megaterium* (CYP106 /102) [5,6,11,12,13,16,17].

RT-PCR product of desired amplicon (1107 bp and 1576 bp) was obtained for CYP3A and CYP2H1, which suggested that PB treatment in chicks caused induction of CYP3A and CYP2H1 genes in Bantam, Bantamized White Leghorn and White Leghorn chicks.

Barbiturates at high dose are well known inducer of CYP3A genes in several species of animals [13]. CYP3A gene was induced in chicken embryos followed by PB treatment. Metyrapone was strong inducer as compared to RU-486, dexamethasone, PCN and rifampicin in chicken [8]. CYP2H1 and CYP2H2 genes were induced in phenobarbital treated chicken embryo hepatocytes. CYP2H1 gene was highly induced by phenobarbital in chicken embryo hepatocytes and it was also found that RU-486 specifically inhibits PB induction of the CYP2H1 gene. Immunodection by a monoclonal antibody of dexamethasone or PCN induced CYP3A like protein in chicken embryo liver was reported by Kimmett *et al.* [14]. This observation suggested existence of an inducible CYP3A like enzyme in chicken. Cloning and characterization of CYP3A37 avian cytochrome suggested that sequence of CYP3A37 was 60 % homologous compare to human CYP3A4. Metyrapone strongly induces CYP3A37 in both *in ovo* and cell culture. As an inducer PB, dexamethasone and PCN are less potent and rifampicin was weak at 50 μM concentration [8]. In the chicken hepatoma cell line, induction occurred in CYP3A37 and the transcript level of CYP3A37 continued to increase regardless of 30 hrs of exposure of 600 μM PB [10]. Elevation of transcript levels of CYP3A37 was also found after treating chicken hepatoma cells for 16 hrs with PB (400 μM), forskolin (100 μM) and 1, 9 dideoxyforskolin (100 μM) [9]. Okaidaic acid inhibits induction of chicken CYP3A37 in chicken hepatoma cells [7,10]. Few reports in other species were also found regarding CYP3A subfamily induction. Steroid 6β-hydroxylase activity was used as marker for CYP3A induction upon PB treatment in dog [1]. CYP3A subfamily gene expression was increased in the liver of PB treated sheep. Treatment of culture hepatocyte with PB resulted in induction of CYP3A12 activity [5].

Quantitative RT-PCR is highly sensitive and reliable technique in quantifying the gene expression. CYP3A was up regulated by a factor of 1.339, 14.507 and 1.004 in Bantam and Bantamized White Leghorn and White Leghorn chicks, respectively. Metyrapone was the most potent inducer of CYP3A showing 3.8 fold inductions much higher than the other classes of xenobiotics inducing CYP3A. RU-486, dexamethasone, PCN and rifampicin induced CYP3A to less than 1.8 fold [8]. CYP3A37 and CYP2H1 expression was elevated after treatment with typical inducers such as metyrapone, PB, dexamethasone, and PCN. Chicken embryos treated with PB (6 mg/egg) and metyrapone (6 mg/egg) *in vivo* resulted in approximately 100 fold CYP3A induction by metyrapone while PB showed only 5 fold induction of CYP3A. Chicken hepatoma cells (*in vitro*) treatment with PB resulted in approximately 15 fold induction of CYP3A whereas metyrapone induced up to 40 fold [17].

Semi quantitative RT-PCR was conducted to assess the induction of CYP3A mRNA using rifampicin and dexamethasone. Rifampicin treatment of HepG2 cells induced CYP3A in a dose and time dependent manner. Cells in culture for 48 hrs with 1 and 50 μM rifampicin showed induction of 2.7 and 5.0 fold in CYP3A mRNA expression in comparison with untreated controls. Graham *et al.* [5] found that rifampicin induced CYP3A4 gene transcription 13 fold *in vitro*. CYP3A37 induction was less at 24 hrs of exposure to chicken embryos with PB but at 48 hrs of exposure, CYP3A37 was strongly and constantly up regulated. This finding suggested that PB had different potencies for induction with different time course profiles. Regulation of CYP2H1 gene expression was similar to CYP3A in chicken. Regulation of CYP2H1 of chicken also resembles to CYP2C of human [17]. The co-induction of CYP3A and multiple drug resistance (MDR1b) genes by dexamethasone in HepG2 cells for 72 hrs, 96 hrs and 120 hrs was studied and it was found that CYP3A and MDR1 genes were induced 5 and 2 folds, respectively. The maximum expression of CYP3A4 occurred after a 72 hrs dexamethasone treatment whereas maximum of MDR1 gene expression was observed after 96 hrs treatment.

Hepatoma cells were treated with PB for 4 hrs and there was an increase of approximately 10 fold in the rate of CYP2H1 gene transcription. This rate is increased to a maximum of 36 fold after 12 hrs of PB treatment but gradually declined to 9 fold at 48 hrs [6]. CYP2H1 is up-regulated 25 fold *in ovo* and *in vitro* chicken hepatoma cells line treatment resulted in 7 fold up regulation [17]. PB and RU-486 moderately elevated CYP2H1 transcript levels approximately 12 fold but metyrapone elevated CYP2H1 transcript levels 18 -25 folds [8].

In conclusion, PB treatment caused induction of CYP3A and CYP2H1 genes in Bantam, Bantamized White Leghorn and White Leghorn breeds of chicks. Quantitative RT-PCR revealed that CYP3A was upregulated in Bantam, Bantamized White Leghorn and White Leghorn chicks. CYP2H1 was upregulated in Bantam, Bantamized White Leghorn chicks, and down regulated in White Leghorn chicks.

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