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Hepatic CYP3A expression and activity in low birth weight developing female rats

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Background: **We aimed to investigate the effects of low birth weight (LBW) on the hepatic expression of cytochrome P-450 3A (CYP3A) in developing female rats.**

Methods: **Pregnant rats were divided into two groups, a nourished group and an under-nourished group. The offspring of the nourished rats were defined as a normal weight, normal diet group (NN group). The offspring of the under-nourished rats were designated as a LBW, normal diet group (LN group). CYP3A mRNA expression, protein expression, protein localization and activity were determined.**

Results: **The CYP3A1 mRNA expression levels of the LN group on days 3, 21, and 56 were significantly higher than those of the same age in the NN group ($P\leq0.01$). The mRNA expression of CYP3A2 in the LN group on day 21 was higher than in rats of the same age in the NN group ($P<0.01$). The staining intensity and frequency of CYP3A1-positive hepatocytes were significantly lower on days 7 and 21 in the LN group than those of rats of the same age in the NN group ($P<0.05$). The staining intensity and frequency of CYP3A2-positive hepatocytes on days 14 and 21 were higher in the LN group than those on the same days in the NN group ($P<0.05$). The mean CYP3A activity of the LN group on day 3 was significantly higher than that of the NN group ($P<0.001$).**

Conclusions: **We found the effect of LBW on CYP3A activity was most prominent during the early days of life in rats. Investigators and clinicians should consider the effect of LBW on CYP3A in both pharmacokinetic study design and data interpretation, when prescribing drugs to LBW infants.**

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Key words: **cytochrome P-450 3A; development; expression; low birth weight**

Introduction

Undernutrition *in utero* could result in low birth weight (LBW) and a range of fetal adaptations and developmental changes.[1,2] These adaptations can lead to permanent alterations in the body structure and metabolism.[3] Changes in physiological, pathophysiological, and/or nutritional conditions often alter the expression of drug-metabolizing enzymes.[4] Every year, approximately 17 million infants with a LBW are born in developing countries.[5]

Cytochrome P-450 3A (CYP3A) subfamily members are the most abundant and clinically important CYP enzymes in the liver. The human and rodent CYP3A forms show expression patterns that are subjected to developmental influences.[6] The CYP3A enzymes are responsible for the maintenance of steroid homeostasis and the metabolism of approximately 50% of the most commonly used drugs.[7-9] Among the major functional CYP3A isoforms, CYP3A4 is the predominant form in humans. CYP3A4 is the primary enzyme involved in catalyzing the metabolism and clearance of a large proportion of clinical medications, including many pediatric drugs. It has been suggested that alterations in the expression or activity of CYP3A isoforms are key predictors of drug responsiveness and toxicity.[10] Our previous study suggested that LBW influenced the hepatic protein expression of CYP3A1 in neonatal rats.[11] However, the effect of LBW on the expression and activity of hepatic CYP3A in developing children and animals remains unclear.

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Murine CYP3A is differentially expressed in the female and male liver.[12] CYP3A1 and CYP3A2 are the rat orthologues of the human enzyme CYP3A4.[13,14] We aimed to investigate the effects of LBW on the expression and activity of CYP3A (CYP3A1 and CYP3A2) in developing female rats. These results will improve our understanding of the developmental changes in CYP3A under maternal undernutrition conditions.

Methods

All procedures used in this study were approved and performed in accordance with the guidelines established by the Animal Ethics Committee of the Zhejiang University School of Medicine.

Animals, feeding protocol and sample preparation

Twelve-week old Sprague-Dawley rats, 2 days of pregnancy (250-300 g), were obtained from Zhejiang Medical Science Academy (Hangzhou, China). The rats were housed in a 22±1ºC environment at 60% humidity and were maintained on a 12h light:12h dark cycle. The number of live pups from each dam varied from 6 to 15. If the dam gave birth to more than 8 pups, only 8 randomly selected pups were permitted to live with their dam. This was done to ensure that each pup received sufficient nutrition through breastfeeding.

Rats were housed individually in standard rat cages. Pregnant rats were randomly divided into two groups, a nourished group and an under-nourished group. Rats in the nourished group were fed *ad libitum*. Meanwhile, rats in the undernourished group were only fed 50% of what the nourished group had, as determined by the amount of food consumed by the nourished group from day 2 of pregnancy until day 23 (parturition). Both groups were fed the same standard commercial rat diet, which contained (per 100 g): protein (22.5 g), carbohydrates (57.0 g), fat (3.9 g), cellulose (8.0 g), minerals (1.0 g), vitamins (mixed, 5.0 g), and water (2.5 g). The metabolizable energy (KJ/100 g) was 1.583. Water was provided to both groups *ad libitum*. All of the rats were fed daily (in the morning), and the food consumption was determined 24 hours later.

During lactation, the dams were fed the same diet that they received during pregnancy. After weaning, all of the pups were housed individually in standard rat cages and fed the same diet as their dams. The dams and pups of each group were fed the same diet throughout the entire experiment.

Groups and sample preparation

The offspring of the nourished rats had birth weights between 5.2 g and 7.2 g. These rats were designated as the normal birth weight-normal diet (standard diet) group. Henceforth, these rats are referred to as a NN group (control group). The offspring of the undernourished rats had birth weights below 5.2 g. These rats were designated as a LBW-normal diet group. Henceforth, these rats are referred to as a LN group (experimental group).

All of the animals were sacrificed by exsanguination under ether anesthesia. At 3, 7, 14, 21, 28, 56, and 84 days after birth, the livers of the female pups from each group were isolated and subsequently examined (n=6-8 per group). The body and liver weights of the pups were recorded, and the hepatic index was calculated accordingly [hepatic index=(hepatic weight/body weight)×100%]. A portion of the right lobe of the liver was fixed in 10% neutral-buffered formalin for immunohistochemical examination. The remainder of each pup's liver was flash frozen in liquid nitrogen and subsequently stored at -80ºC until it was subjected to RNA and microsome extraction.

RNA extraction and real-time PCR

RNA extraction, gene-specific primers and real-time PCR were used in our previous studies.[11,15,16] Total RNA was extracted from liver tissue using the Total RNA Miniprep Kit (Axygen Biosciences,CA 94587,USA) according to the manufacturer's protocol. Gene-specific primers were designed according to the sequences deposited in GenBank. PCR reactions were performed twice in duplicate using a 7500 Applied Biosystems instrument. To analyze the results after real-time RT-PCR experiments, we quantified the target gene transcript relative to that of the references gene transcript, GAPDH, using the ΔCT method ($2^{CT[GAPDH]-CT[target]}$), as described by the Bio-Rad real-time PCR protocol.

Immunohistochemistry

The immunohistochemical analysis of CYP3A1 and CYP3A2 proteins in the paraffin-embedded liver sections were performed as in our previous studies.[11,15,16] The CYP3A1 and CYP3A2 proteins were detected using a two-step immunohistochemical technique with the DAKO Envision system. The sections were stained with peroxidase-diaminobenzidine and then counterstained with hematoxylin. Images were captured with a digital camera mounted to an Olympus microscope.

Samples were analyzed by calculating the percentage of positive cells in five fields under a microscope. Protein expression was evaluated using a score corresponding to the sum of both (a) staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and (b) percentage of positive hepatocytes (0, 0% positive; 1, <25% positive; 2, ≥25 and ≤50% positive; 3, >50% positive). Using summed score

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values (maximum possible=6), samples were classified as "-" (0), "+" (1-2), "++" (3-4) and "+++" (5-6). A score greater than 2 was regarded as positive.[17] Each sample was scored in duplicate by two blinded observers.

Assay of CYP3A activity

The assay of CYP3A activity with 3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-methylsulfonyl) phenyl] furan-2(5H)-one (DFB, Merck Frosst, Que, Canada) is described in our previously published work.[15] The rat liver microsomes were prepared by calcium precipitation as previously described.[18] The microsomal protein concentration was determined by bicinchoninic acid (BCA) assay (Beyotime Institute of Biotechnology, Jiangsu, China). DFB is specifically metabolized by human CYP3A4 and is debenzylated to 3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl] furan-2(5H)-one (DFH) by liver microsomes. DFH could be easily measured by fluorescence. The production of DFH was used as a measure of the microsomal CYP3A activity based on previously published methods.[19] The final production of DFH was quantified with a standard curve made with pure DFH (Merck Frosst, Que, Canada).

Statistical analysis

Statistical analyses were performed with R software 2.6.0. Differences between the groups for the mRNA and protein expression levels were determined by an independent-sample *t* test or the Mann-whitney *U* test as appropriate. For multiple comparisons, the Dunnett test was used to statistically examine the differences in the mean values. A *P* value less than 0.05 was considered significant in all cases.[15]

Results

Body weight, hepatic weight and hepatic index

The average body weight of the LN group throughout the 12 weeks after birth was lower than that of the NN group, with significant differences observed on days 3, 7, 28, and 56 (Table). The average hepatic weight of the LN group during the whole developing period was also lower than that of the NN group, with significant differences observed on days 7, 28, and 56. The hepatic index (liver weight per unit of fetal weight) on day 3 was significantly higher in the LN group than in the NN group.

mRNA expression of CYP3A1 and CYP3A2

The mRNA expression levels of CYP3A1 in the LN group on days 3, 21, and 56 were significantly higher than those of rats of the same age in the NN group ($P \leq 0.01$). The varied mRNA expressions of CYP3A1 in the NN group and LN group were dependent on the age of the rats (Fig. 1). The mRNA expression of CYP3A1 in the LN group reached a higher level on day 21. The expressions were higher on days 21 and 28 than on day 84 in the LN group ($P<0.05$) (Fig. 1B). In the NN group, however, the mRNA expression gradually increased from day 3 to day 14 and peaked on day 14. The expression levels in the NN group on days 14, 21, and 28 were similar and began to decline on day 56. There were significant differences on days 3, 14, 21, 28, and 56 compared with day 84 under normal birth weight conditions (Fig. 1A).

The expression levels of CYP3A2 in the LN group on day 21 were higher than those of rats of the same age in the NN group ($P<0.01$). The expression of CYP3A2 in both groups varied with age (Fig. 1C&D). CYP3A2 expression in the LN group peaked days 14 to 28 and decreased to its lowest level on day 56 (Fig. 1D). Meanwhile, CYP3A2 expression in the NN group reached a peak level on day 14 (Fig. 1C). The expression in the LN group on days 56 and 84 was extremely low, similar to what was observed in the NN group.

Effects of LBW on the protein expression of CYP3A1 and CYP3A2

CYP3A1 protein expression in LBW rats

CYP3A1 immunolabeling was observed in the cytoplasm of hepatocytes (Fig. 2). There was no significant difference in zonal expression pattern between the NN and LN groups. CYP3A1 was expressed diffusely throughout the hepatocytes from day 3 to day 21 in all of the groups (Fig. 2). On day 28 and after, an obvious zonal expression pattern (with a stronger staining intensity of the hepatocytes around the central vein) appeared in the NN and LN groups. At this time, the staining intensity became gradually weaker from the perivenous to the periportal hepatocytes.

The CYP3A1 expression score of the LN and NN groups peaked on day 14. CYP3A1 expression on day 14 was significantly higher than that on day 84 ($P=0.037$ for the LN group, $P=0.004$ for the NN group). The protein expression level of CYP3A1 in the LN group on day 3 was significantly higher than that observed on day 84 ($P=0.026$).

The staining intensity and frequency of CYP3A1 positive hepatocytes were significantly lower on day 7 and day 21 in the LN group compared with rats of the same age in the NN group.

CYP3A2 protein expression in LBW rats

The localization pattern for CYP3A2 was similar to that for CYP3A1. Immuno-labeling was evident in the

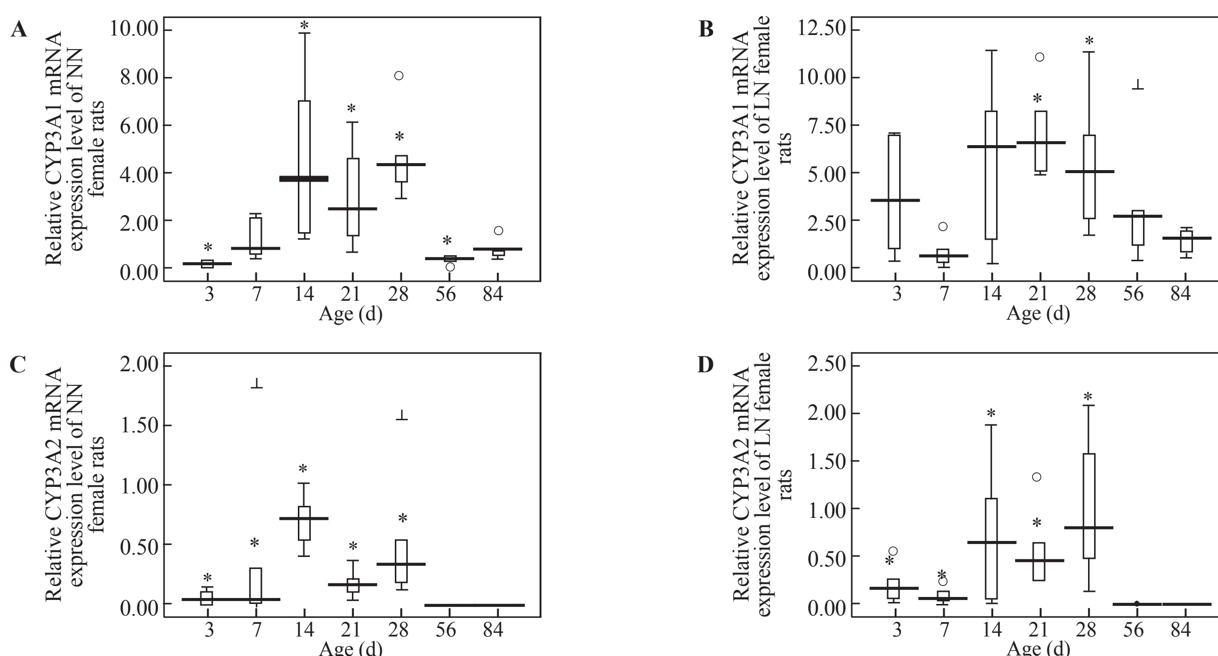

Fig. 1. Ontogeny of the cytochrome P-450 3A1 (CYP3A1) (**A&B**) and CYP3A2 (**C&D**) mRNA expression. Ontogeny of the CYP3A1 (**A&B**) and CYP3A2 (**C&D**) relative mRNA expression levels in the NN (normal weight, normal diet group) and LN (low birth weight, normal diet group) groups (n=6/group) as assessed with real-time PCR. The values were normalized against GAPDH. The data are presented as box-and-whisker plots (boxes represent the 25th and 75th percentiles, heavy bar represents the median). The whiskers represent the 10th and 90th percentiles. *: $P<0.05$, *vs.* values on day 84. ○: each data point that is more than 1.5 times the interquartile range; ⊥: outliers that are more than 3 times the interquartile range.

cytoplasm of the hepatocytes. There were no zonally restricted CYP3A2 expression patterns from day 3 to day 21 in any of the groups (Fig. 3). CYP3A2 was expressed diffusely throughout the hepatocytes. On day 28 and later, an obvious zonally restricted expression pattern was observed in the NN and LN groups (Fig. 3). There was a stronger staining intensity in the hepatocytes around the central vein, and the staining intensity became gradually weaker from the perivenous to periportal hepatocytes. No CYP3A2-positive hepatocytes were observed on days 56 and 84 in both groups (Fig. 3).The staining intensity and frequency of CYP3A2-positive hepatocytes in the LN group on days 14 and 21 were higher than those in the NN group on the same days ($P<0.05$).

CYP 3A activity

DFH production in rat liver microsomes is shown in Fig. 4. The mean activity was the strongest for both weight groups on day 21.

The production of DFH in the NN group was significantly different on days 3, 7, and 21 when compared with day 84 (Fig. 4). The activities in the LN group on days 3, 21, 28, and 56 were significantly different when compared with the activity on day 84 (Fig. 4).

The mean DFH production in the LN group on day 3 was significantly higher than in the NN group ($P<0.001$).

Discussion

This study demonstrated that LBW had consequences on body and hepatic weights. Additionally, LBW had influences on hepatic CYP3A mRNA and protein expression in developing female rats, while its effects on different ontogeny periods were different. The effect of LBW on CYP3A activity was most prominent during the first days of life.

In vitro and *in vivo* studies indicated that profound changes occur in the activity of CYP3A isoforms during all stages of development.[20] Understanding the developmental changes of CYP3A under different pathological and physiological conditions is one of the key factors that determines the pharmacokinetic status in postnatally developing individuals.[21] To the best of our knowledge, this is the first report that details the developmental changes of hepatic CYP3A1 and CYP3A2 expression and activity under LBW conditions.

Nutrition is the major intrauterine environmental factor that alters the expression of the fetal genome, and it may have lifelong consequences. Specifically, alterations in fetal nutrition and endocrine status may result in developmental adaptations that permanently change the structure, physiology, and metabolism of the offspring.[22] The liver is the major organ for drug biotransformation and elimination. Therefore,

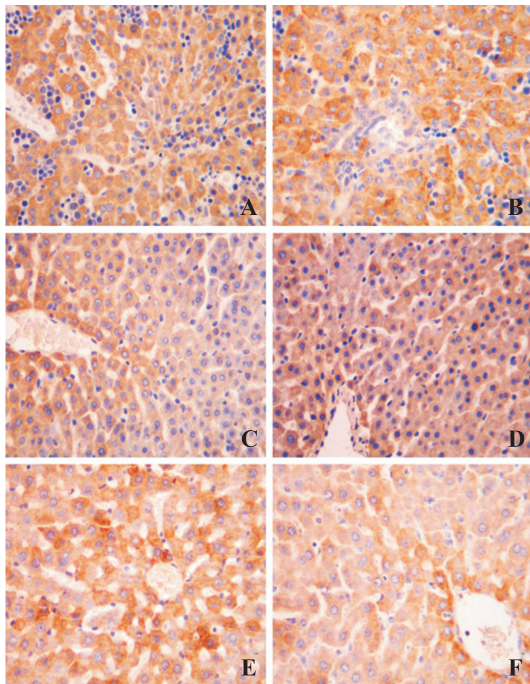

Fig. 2. Photomicrographs of hepatic sections that were immunostained for cytochrome P-450 3A1 (CYP3A1) from developing rats. Photomicrographs of hepatic sections from developing rats of different birth weights and diet groups that were immunostained for CYP3A1. Representative images from day 3 (**A&B**), day 28 (**C&D**) and day 84 (**E&F**) in the NN (normal weight, normal diet group, left column) and the LN groups (low birth weight, normal diet group, right column) are shown. Protein staining is shown in brown, and hematoxylin counterstaining is shown in blue (original magnification × 200).

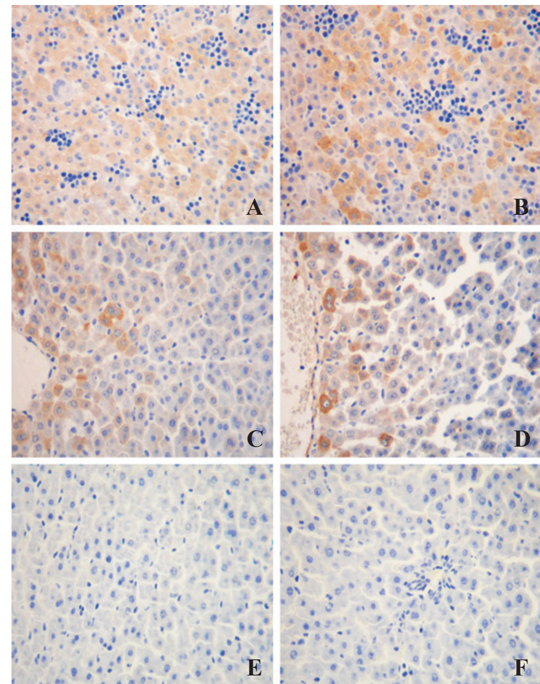

Fig. 3. Photomicrographs of hepatic sections that were immunostained for cytochrome P-450 3A2 (CYP3A2) from developing rats. Photomicrographs of hepatic sections from developing rats of different birth weights and diet groups were immunostained for CYP3A2. Representative images from day 3 (**A&B**), day 28 (**C&D**) and day 84 (**E&F**) in the NN (normal weight, normal diet group, left column) and the LN groups (low birth weight, normal diet group, right column) are shown. Protein staining is shown in brown and hematoxylin counterstaing is shown in blue (original magnification × 200).

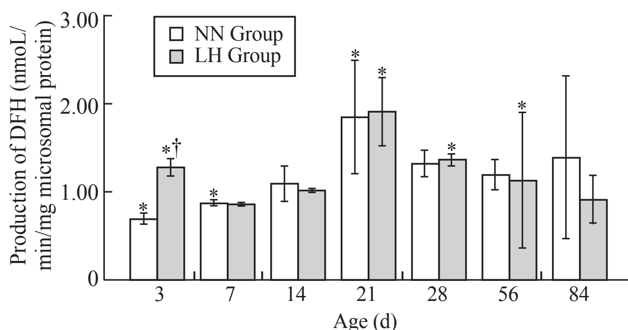

Fig. 4. The production of DFH by rat liver microsomes incubated with DFB. The error bars indicate the standard deviation (n=3-4). *: P<0.05, *vs*. day 84 of the group with the same diet; †: P<0.05, *vs*. rats of the same age in the NN group. NN group: normal weight, normal diet group; LN group: low birth weight, normal diet group.

any relative decrease or increase in its size could have functional implications for the metabolism and elimination of drugs in children.[23] This study demonstrated that the average body and hepatic weights of the LN group throughout the developmental period were lower than those of the NN group. Significant differences were observed on days 3, 7, 28, and 56 for body weight and days 7, 28, and 56 for hepatic weight.

Previous studies suggested that adaptive changes could happen in fetal organ development in response to maternal and fetal malnutrition. Essential organs such as the brain and lungs are relatively protected from reductions in growth at the expense of visceral organs such as the liver.[24] Our study further indicated that LBW might have consequences on the body weight and hepatic weight of developing female rats. These changes can continue to the period of adulthood without postnatal nutrition intervention. Although no significant differences in the body weight were found on day 3, the hepatic index (liver weight per unit fetal weight) in the LN group was significantly higher than what was observed in the NN group. This result was similar to a previous study, which indicated that fetuses from nutrient-restricted ewes were markedly lighter than those from control-fed ewes.[25] However, these nutrient-restricted fetuses exhibited higher liver weights per unit of fetal weight than the control fetuses.[25] The increased liver weight per unit of fetal weight in fetuses from nutrient-restricted pups compared with control-fed pups may be explained by an increase in liver metabolic activity, which is imperative for development. Our study revealed that the hepatic index, mRNA expression of

CYP3A1 and activity of CYP3A in the LN group on day 3 was significantly higher than those observed for rats of the same age in the NN group. These data indicated that the hepatic index, mRNA expression of CYP3A1 and activity of CYP3A of the LN group on day 3 in fetal/placental development may be beneficial to early fetal survival in the face of nutrient restriction.[25]

Our study found that CYP3A1 mRNA expression levels in the LN group on days 21 and 56 were significantly higher than those of rats of the same age in the NN group. However, higher CYP3A1 mRNA expression did not lead to higher protein expression levels in the LN group. Rats weaned around day 21 and developed very quickly during the period of puberty (day 56).[26] As CYP3A is involved in the metabolism of steroids and numerous xenobiotics,[27] we hypothesized that CYP3A1 in the LN group on days 21 and 56 was up-regulated in hepatic cells to sustain xenobiotic metabolism and hormone homeostasis. However, the CYP3A1 gene in LBW rats was less stable than in NBW rats. The discrepancy in mRNA and protein of CYP3A1 suggests a short birth–death evolution of mRNA in the LN group and post-translational modification.

The significantly lower staining intensity and frequency of CYP3A1 positive hepatocytes were observed on days 7 and 21 in the LN group when compared with rats of the same age in the NN group. This result further indicated that the post-translational modification of CYP3A1 mRNA expression in LBW rats is not the same as in NBW rats especially during the early period of time. Our study also revealed that CYP3A2 mRNA expression in the LN group on day 21 was significantly higher than that observed in rats of the same age in the NN group. The staining intensity and frequency of CYP3A2-positive hepatocytes in the LN group on days 14 and 21 were higher than those in the NN group on the same days. CYP3A1 and CYP3A2 are known to be differentially regulated during development.[14] Furthermore, the CYP3A2 promoter binds a different set of transcription factors compared with the CYP3A1 promoter.[28] There is increasing evidence that maternal nutritional status can alter the epigenetic state (defined as stable alterations of gene expression through DNA methylation and histone modifications) of the fetal genome.[22] The results from this study indicate that further studies are needed to examine the epigenetic modification of CYP3A and its related regulation of gene expression under LBW conditions.

No significant activity differences between the LN and NN groups were found during the developmental period except on postnatal day 3. A previous study suggested that ethylmorphine N-demethylation activity (a marker for CYP3A activity in rat hepatic microsomes) was significantly lower in newborn males with low body masses. However, this was fully developed as early as the 5th postnatal day.[29] The findings in this study are similar to ours and indicate that the postnatal adaptation of rats with growth retardation at birth was delayed especially during the first days of life.[29]

Our study found that the peak level of CYP3A1 mRNA expression occurred later in the LN group than in the NN group (day 21 *vs.* day 14). CYP3A2 mRNA expression in the LN group was higher on days 14 to 28, whereas it peaked at day 14 in the NN group. These data indicated that the time until maturity of the drug-metabolizing enzyme in LBW rats was different from that in normal birth weight rats. It was interesting to find that the CYP3A1 expression scores of the LN and NN groups were the highest on day 14, whereas the mean activity was the strongest for both weight groups on day 21. These data suggested that there was a discrepancy between protein expression and activity, which indicated that a complex posttranslational modulation may regulate CYP3A function.

Given the prominent role of CYP3A in drug metabolism, CYP3A is inducible by many of its substrates, its CYP3A expression changes during pediatric development are highly variable among individuals.[6,30] As a result, one limitation of this study is that even though 6-8 samples in this study are large enough for animal study but may not stand for the whole induction and inhibition situation in different developmental periods. The other limitation of this study is that we only examined hepatic CYP3A expression in female rats. The effects of LBW on developing male rats and the function of other CYP3A substrates in both sexes require further research.

In conclusion, this study examined hepatic CYP3A expression and activity in developing female rats under LBW conditions. The results showed that LBW had consequences on the body and hepatic weights of developing female rats. The effect of LBW on CYP3A activity was most prominent during the first days of life. Our findings suggested that investigators and clinicians should consider the effect of LBW on CYP3A in pharmacokinetic study design and data interpretation, or when prescribing drugs to LBW young children.

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