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Original Article

Impact of perinatal exposure to acetaminophen on hepatocellular metabolic function in offspring

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Abstract: Acetaminophen (APAP), an over the counter (OTC) medication, is widely used in antipyretic treatment. Although the risk of dose-dependent cytotoxicity has been known, the potential effect of perinatal exposure to acetaminophen on metabolic function in offspring remains uninvestigated. Therefore, we established a prenatally APAP-exposed pregnancy mouse model to assess the possible adverse effect on liver metabolic function in offspring. Biochemical assays were applied in analysis of basic metabolic parameters in postnatal mice. Further, immunoblotting assay was used to assess the expressions of insulin receptor β (IRβ), insulin receptor substrate 1 (IRS1), phospho-Akt and phospho-GSK-3β proteins in liver cells. In addition, hepatic glucose transporter 2 (GLUT2) immunoactivity was determined by using immunohistochemistry staining. Compared with untreated postnatal mice, APAP-exposed offspring induced impaired glucose metabolism, increased plasma insulin level, and reduced liver glycogen content. In addition, APAP exposure decreased the expressions of IRS1 and phospho-GSK-3β, phospho-AKT proteins and down-regulated the level of glucose-import regulator GLUT2 in the liver. Taken together, our preliminary findings indicate that perinatal APAP exposure-impaired hepatic glucose metabolism in offspring may be associated with disturbance of insulin-dependent AKT signaling in the liver.

Keywords: Acetaminophen, perinatal exposure, liver, glucose metabolism

Introduction

Acetaminophen (APAP) is commonly used as an antipyretic medication in clinical prescription. However, time- and dose-dependent side effects induced by APAP is of specific concern [1]. Notably, APAP can trigger dosed hepatotoxicity beyond therapeutical range, accompanied with other adverse complications [2]. The recommended dose of APAP is maximal use of 325 mg per day, as issued by Food and Drug Administration of United States [3]. Thus, APAP is generally considered to be safe and it is commonly self-medicated by pregnant woman [4]. Some potential threats of APAP to pregnancy have been reported, such as miscarriages, preterm birth, and prenatal APAP exposure is related to poor health in fetal development [5, 6]. In pharmacokinetics, dissociative APAP can be transferred to fetus via placenta, implying the potential health risk to offspring [7]. As limited, there is less published information regarding the prenatal exposure of APAP on hepatic function in offspring, especially metabolic homeostasis. Further, investigating on whether prenatal exposure to APAP may affect liver metabolism-regulated key pathways remains unknown. In our current study, pregnant mice were subjected to treatment with recommended dose of APAP before parturition. Correspondingly, offspring mice in postnatal day 21 (PND 21) were used to conduct molecular and biochemical assays for hepatic metabolic functions, and were further assessed the possible effect of APAP on glucose metabolism and target protein transcript associated with key pathway.

Materials and methods

Experimental reagents

High purity of APAP (≥99%) was obtained from YUANYE BioTechnology Co., Ltd. (Shang-

hai, China). As described, other required chemicals/kits were showed as follows.

Animal desgin and treatment

Health mature female and male Kunming (KM) mice, aged 8-week-old (20-25 g), were purchased from the Experimental Animal Centre of Guangxi Medical University (Nanning, China). Animal processes used were conducted in accordance with the protocols of *Institutional Ethical Committee* of Guangxi Medical University.

After acclimation for one week, mice were designated to be copulated, and female mouse will be checked the vaginal plug for pregnancy confirmation on next day. Each copulated mouse was housed individually and was settled down under controlled environments. The pregnant mice were treated with 300 mg APAP/kg body weight dissolved in phosphate buffered saline twice, through oral gavage during gestational day 13 and 14. The control mice were intragastrically given the same volume of phosphate buffered saline. At the end of weaning period, the F1 offspring mice were sacrificed on postnatal day 21 (PND 21) after glucose tolerance testing, and the plasma sample and liver tissue were harvested for further experiments.

Oral glucose tolerance test (OGTT)

All mice on PND 21 were fasted for 16 h before the following experiment. The fasting blood glucose level was measured by using an ACON-Biotech glucometer (Hangzhou, China). After the initial blood glucose level being determined, 2 g/kg body weight glucose solution was orally given to the mice and blood glucose was measured within 15, 30, 60, 90 and 120 min, respectively.

Serum and metabolic parameters

Serological contents of alanine aminotransferase (ALT), glucose and hepatic glycogen level followed by APAP prenatal exposure were assayed by using the commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in according to the manufacturer's instructions. The fasting insulin concentration was tested by an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Elisa Biotech Inc., China).

Routine and immunohistochemical analysis

As reported previously [8, 9], fresh liver sample was fixed with 10% neutral formalin and prepared as paraffin-embedded block, then was further processed as 5 μm section. Subsequently, the slices were stained with hematoxylin and eosin.

After being subjected to deparaffinized and rehydrated steps, other sections were blocked with 10% BSA for 1 h at room temperature. Then, the sections were incubated with rabbit-anti-glucose transporter 2 antibody (1:500; Boster, Wuhan, China) overnight at 4°C, followed by horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (1:1000; Boster, Wuhan, China) for 1 h at room temperature. Accordingly, chromogenic diaminobenzidine (DAB) was developed as binding to HRP substrate prior to nucleus being counterstained with haematoxylin. The sections were mounted and imaged, as well as data analysis.

Western blot assay

Freshly extracted liver protein was prepared by using RIPA lysis buffer (Beyotime, China) supplemented with 1 mM protein inhibitor (PMSF) (Beyotime, China). Hepatic protein concentration was determined by using an Enhanced BCA Protein Assay Kit (Beyotime, China). The protein (40 μ g per lane) was separated through running 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). Membrane was blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature, followed by incubated with diluted primary antibodies (1:500, rabbit anti insulin receptor beta (IRβ), insulin receptor substrate 1 (IRS1), phospho-GSK-3β, phospho-Akt) at 4°C overnight. After washing with tris-buffered saline containing 0.1% tween 20 (TBST), membrane was incubated with horseradish peroxidase-coupled secondary antibodies (Beyotime, China) for 1 h at room temperature. Band on membrane was developed by using an enhanced chemiluminescence (ECL) detection kit (Beyotime, China) and was visualized by exposure to X-OMAT BT film (Kodak, NY, USA). Beta-actin was used as internal control when being normalized and quantified with samples in analyzing optical density by Image J software (NIH, USA).

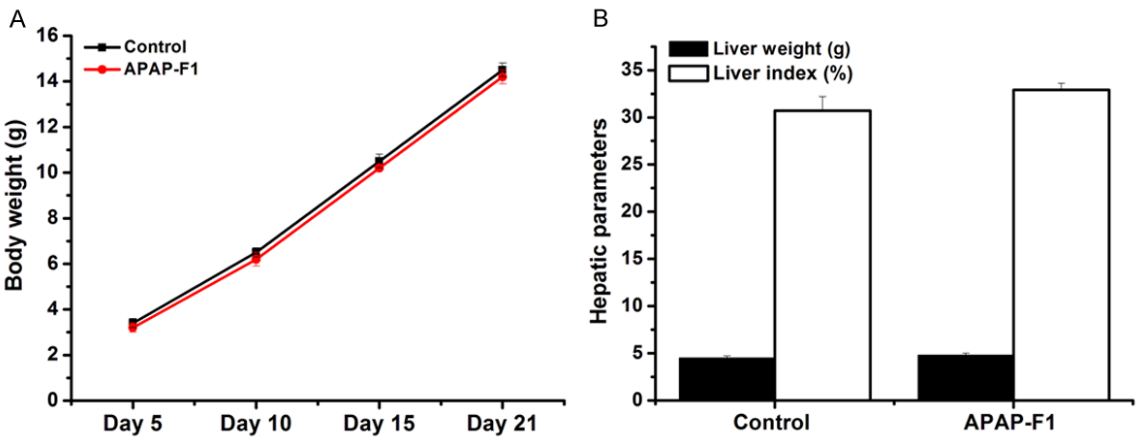

Figure 1. *Effect of preinatal APAP exposure on vital sign in F1 offspring.* Statistical data was analyzed by using SPSS 19.0 software. Differences between two-compared groups were assessed by a one-way analysis of variance (ANOVA) followed by Student's. Result was expressed as mean ± SD. Notes: vs. Control, $^{a}P<0.05$; APAP-F1 = preinatal APAP exposure in F1 offspring.

Statistical analysis

Statistical data were analyzed by using statistical product and service solutions (SPSS) 19.0 software. Differences between two compared groups in biochemical and molecular assays were assessed by a one-way analysis of variance (ANOVA) followed by Student's t test. Result was expressed as mean ± SD. A *P* less than 0.05 was considered as statistically significant.

Results

Effects of preinatal APAP exposure on glucose metabolism in offspring

During the designated postnatal days, all offspring mice were recorded the body weights. However, they did not show statistical significance on body weight, liver mass and related liver index (**Figure 1A**). In prenatal exposure APAP group, increased trend in the liver index and alanine aminotransferase (ALT) of the PND 21 offspring was observed, but there were no significant difference when compared to control mice (**Figure 1A-C**).

Significant impacts on fasting glucose and plasma insulin concentration were exhibited in the PND 21 mice. OGTT data indicated that the blood glucose was increased in the prenatal APAP-exposed F1 mice (P<0.05) (**Figure 2A**). The fasted blood glucose contents of prenatal APAP-exposed F1 mice were lower than that in respective control group (P<0.05) (**Figure 2D**). As well, the fasted blood insulin level in prenatal APAP-exposed F1 mice were increased as compared to the control mice (P<0.05) (**Figure 2E**).

To validate the disposal of extrahepatic glucose, hepatic glycogen content was determined. As a result, prenatal APAP-exposed F1 mice showed the reduced liver glycogen when compared to that in unexposed F1 mice (P< 0.05) (**Figure 2B**).

Effects of preinatal APAP exposure on glucose uptake in offspring

To investigate possible effect of APAP exposure on hepatic glucose consumption, key regulator of glucose transporter 2 (GLUT2) in liver cells was assayed by using immunohistochemistry (IHC). As shown in **Figure 3**, the positively GLUT2-labeled liver cells were observed, in which intrahepatic GLUT2 positive cells in preinatal APAP exposure F1 mice were decreased significantly, when compared to those in control (*P*<0.05).

Effects of preinatal APAP exposure on the AKT signaling and associated substrate in offspring

Based on the effects observed, further validation of molecular mechanism was assessed via using western blotting method. The obvious reduction of insulin receptor β (IRβ), a transmembrane IR subunit, was observed in liver tissue

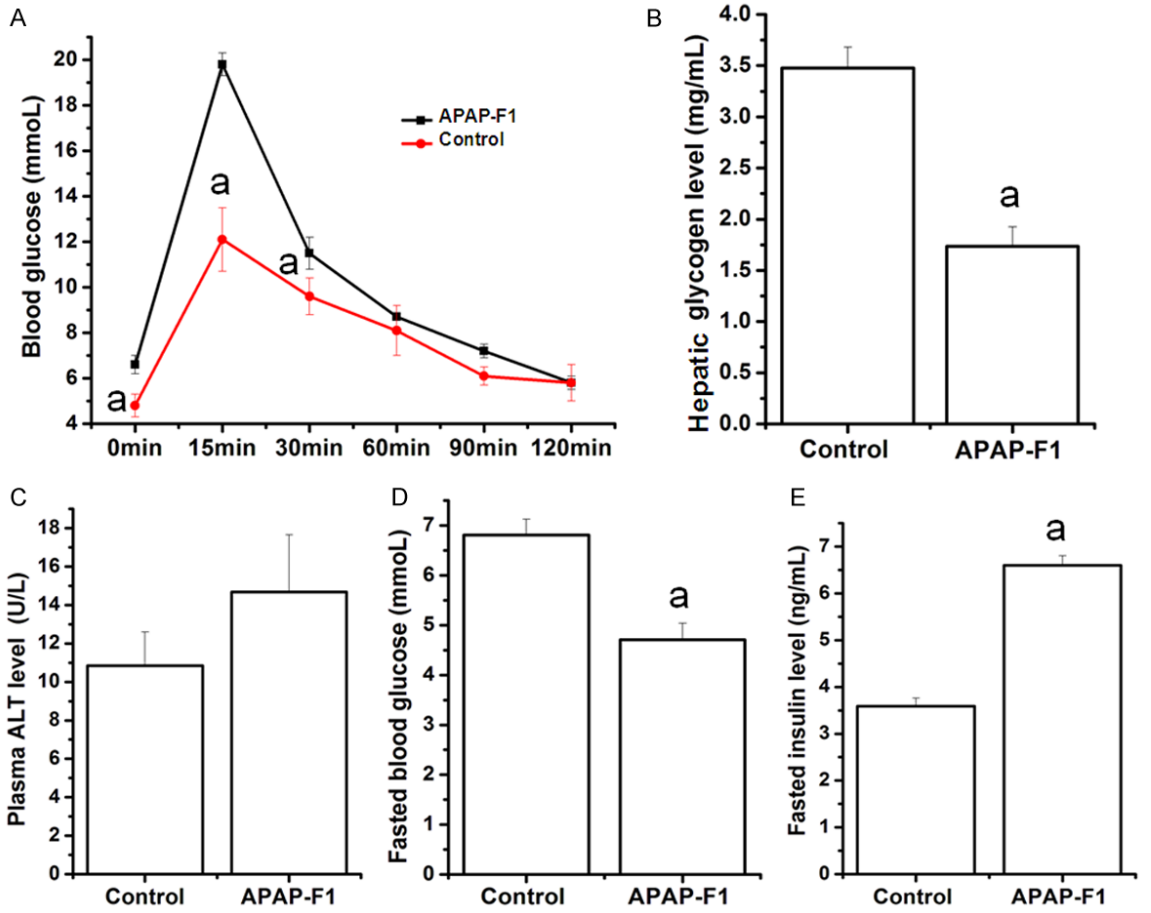

Figure 2. *Effect of preinatal APAP exposure on glucose metabolism in F1 offspring.* Statistical data was analyzed by using SPSS 19.0 software. Differences between two-compared groups were assessed by a one-way analysis of variance (ANOVA) followed by Student's. Result was expressed as mean ± SD. Notes: vs. Control, $^{a}P<0.05$; APAP-F1 = preinatal APAP exposure in F1 offspring.

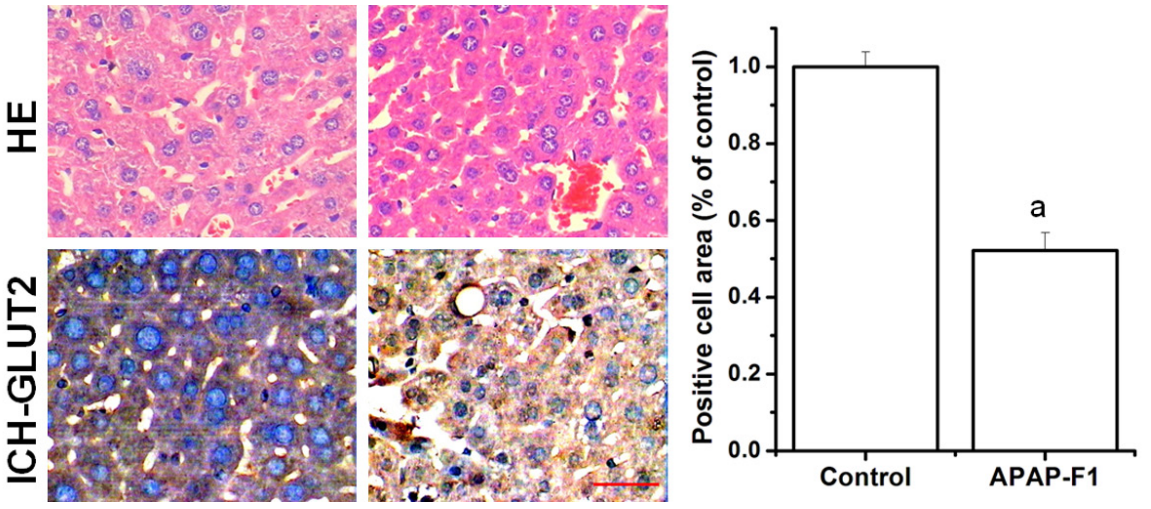

Figure 3. *Effect of preinatal APAP exposure on GLUT2 expression in the liver* (Immunohistochemistry stain, scale bar = 200 μm). Statistical data was analyzed by using SPSS 19.0 software. Differences between two-compared groups were assessed by a one-way analysis of variance (ANOVA) followed by Student's. Result was expressed as mean ± SD. Notes: vs. Control, $^{a}P<0.05$; APAP-F1 = preinatal APAP exposure in F1 offspring.

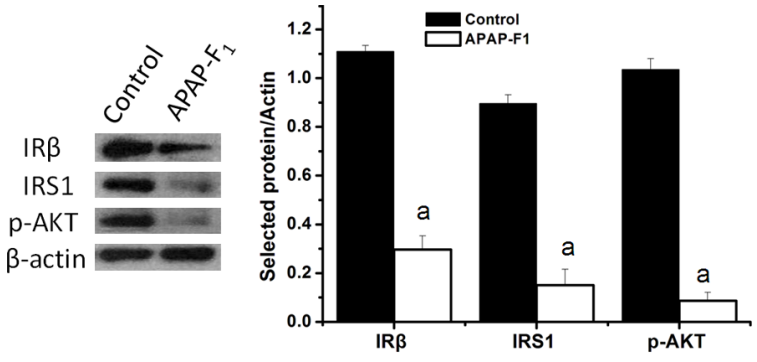

Figure 4. *Effect of preinatal APAP exposure on the AKT signaling in the liver*. Statistical data was analyzed by using SPSS 19.0 software. Differences between two-compared groups were assessed by a one-way analysis of variance (ANOVA) followed by Student's. Result was expressed as mean ± SD. Notes: vs. Control, [a]*P*<0.05; APAP-F1 = preinatal APAP exposure in F1 offspring.

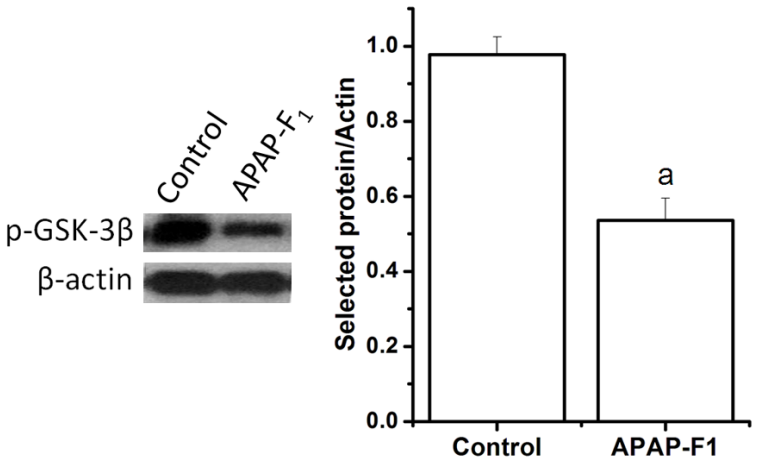

Figure 5. *Effect of preinatal APAP exposure on the AKT-associated substrate in the liver.* Statistical data was analyzed by using SPSS 19.0 software. Differences between two-compared groups were assessed by a one-way analysis of variance (ANOVA) followed by Student's. Result was expressed as mean ± SD. Notes: vs. Control, [a]*P*<0.05; APAP-F1 = preinatal APAP exposure in F1 offspring.

after preinatal exposure to APAP (*P*<0.05). The protein level of insulin receptor substrate 1 (IRS-1) was significantly upregulated after APAP preinatal exposure (*P*<0.05). Notably, the phosphorylation of AKT was significantly reduced in the livers of APAP-F1 when compared to that in unexposed control (*P*<0.05) (**Figure 4**).

In order to characterize AKT substrate involved, glycogen synthase kinase 3-beta (GSK3β), a downstream target of AKT, was selected for determination. As the consequence, preinatal exposure to APAP in F1 mice showed a significant increase in phosphorylated GSK3β protein when compared to that in control (*P*<0.05) (**Figure 5**).

Discussion

In clinical guide, patients using recommended dosage APAP up to 4 g daily need to be monitored the outcome of pan-organ toxicity [10, 11]. Notably, APAP can induce serious liver impairment when overdose is administered [12]. During pregnancy, APAP is the most commonly-prescribed medicine because of it being considered to be safe [13]. Further, the possible adverse effect of using APAP has not been well studied in pregnant women and offspring (generation effect) [14]. Hepatocytes are metabolic controllers in the body, in which play critical roles in maintenance of insulin-dependent glucose homeostasis [15]. Since increasing reports have indicated APAP-mediated hepatic dysmetabolism, thereby, we aim to investigate whether perinatal APAP exposure would affect susceptibility of liver metabolic function in the offspring. In the current study, the rodent model of perinatal exposure to APAP was used to explore the potential effect of APAP on glucose metabolism in offspring. As a result, the biological effects induced by APAP showing impaired glucose tolerance, and reduced insulin concentration, hepatic glycogen content were observed in postnatal mice, implying that APAP may serve as the possible chemical stressor during fetal development in metabolic functions. However, the molecular mechanism behind these actions warrants to be further investigated.

Functionally, liver exerts an important role in maintaining blood glucose level via normalizing

glucose storage and release in glycogenesis or gluconeogenesis [16], respectively. Biologically, APAP can pass through placenta to induce developmental impact on fetus. Glucose transporter 2 (GLUT2) represents a transmembrane carrier protein that controls extracellular glucose to across cell membranes [17]. To decipher underlying mechanism responsible for impaired blood glucose, glucose-sensitized GLUT2 expression was evaluated in postnatal mice (F1). The alteration of GLUT2 indicates impaired liver function in glucose metabolism, in which the downregulation of hepatic GLUT2 expression might be linked to induction of physiological changes to insulin responsiveness.

To further characterize the effects of perinatal APAP exposure on glucose homeostasis, we assayed the key effector of glucose-regulated proteins in this report. In accordance with the change of the AKT signaling pathway after APAP exposure, offspring mice showed a trend of developing glucose dysregulation. AKT (also called PKB) is activated when it responds to insulin and growth factors by phosphoinositide 3-kinase (PI3K)-dependent manner [18]. Insulin mediates a group of vital biological events, especially stimulating disposal of blood glucose in responsive tissues, such as liver that extracellular glucose is oxidised or stored as energy or glycogen [19]. In addition, glycogen synthase kinase-3 (GSK-3) represents serine-threonine kinase that is initially identified as phosphorylation and inactivation of enzyme in glycogen synthase [20]. As the results, elevated phosphorylation of AKT and GSK3β was observed in the livers of APAP-exposed F1 mice when compared to those of the control mice. Moreover, both insulin receptor β and insulin receptor substrate 1 protein expressions were impaired after prenatal exposure to APAP. These results suggested that APAP-affected glucose metabolism and insulin sensitivity in F1 mice might be linked to insulin-dependent AKT pathway.

Conclusions

Our present report may be the first preliminary study to investigate the underlying mechanism of prenatal exposure of APAP on glucose metabolism in the liver. Our findings indicate that prenatal exposure to APAP in offspring may affect AKT signaling responsible for insulin-dependent glucose metabolism in liver cells. However, as limitation in this report, further investigation warrants to be demonstrated the potential association between prenatal exposure to APAP and functional changes in insulin-produced pancreas and insulin-responsive tissues (liver, muscle and adipose).

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Disclosure of conflict of interest

None.

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