Protective role of heptamethoxyflavone against acetaminophen induced hepatotoxicity in zebrafish

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Paracetamol [acetaminophen (APAP)] toxicity is universal drug induced basis of acute hepatotoxicity. Here, we have induced hepatotoxicity in zebrafish model using acetaminophen and studied protective role of heptamethoxyflavone isolated from the weed Sphaeranthus amaranthoides Burm.f. Acetaminophen depletes antioxidants such as GST, SOD and CAT levels, elevated the hepatic marker enzymes (ALT and AST), induced apoptosis, and caused hepatocyte necrosis. Heptamethoxyflavone treatment showed protective role on zebrafish embryos as well as adults. It enhanced the levels of antioxidant enzymes and normalized the hepatic markers significantly compared the control and acetaminophen induced groups. While the acetaminophen group showed abnormal levels of Bcl-2, bax, TNF-α and caspase-3 in the heptamethoxyflavone treated group showed significant increase in Bcl-2 levels and reduced levels of apoptotic gene expression (bax, TNF-α and Caspase-3). Histological observations with H & E staining in heptamethoxyflavone treated group showed normal texture of liver cells. These observations confirm the protective role of heptamethoxyflavone isolated from Sphaeranthus amaranthoides against the acetaminophen induced hepatotoxicity.

Keywords: APAP, BCL2, Danio rerio, Hepatocyte necrosis, Paracetamol

Acetaminophen toxicity occurs by formation of noxious NAPQI metabolite. Some amount of acetaminophen is metabolized by hepatic cytochrome CYP 2E1 via phase I oxidation and result in a highly toxic intermediate, N-acetyl-para-benzo-quinone imine (NAPQI). Excess quantities of NAPQI leads to depletion of GSH, which causes oxidative stress and mitochondrial dysfunction making suppression in Adenosine triphosphate (ATP) stores. Metabolic activation of acetaminophen produces metabolite NAPQI that binds to number of proteins, especially to mitochondrial proteins. Binding of NAPQI to mitochondrial proteins leads to GSH, SOD and CAT depletion and thereby alters the levels of mitochondrial enzymes. The conjugated metabolite alters the mitochondrial ATP-synthase α, subunit, leading to ineffective ATP production. Overdose of aceta-minophen also triggers the transcriptional factors for Bax, caspase-3 and TNF-α and the hepatic levels of these transcriptional factors are consistently raised during acetaminophen induced toxicity and associated with histological severity.

Polypheholic compounds such as flavonoids exhibit a potential to penetrate through the lipid protein bilayers which is important for enabling protection against oxidation of the enzymes. Natural antioxidants, such as heptamethoxyflavones can inhibit the propagation of lipid oxidation by two mechanisms: (i) by intercepting intra-membrane radicals; and/or (ii) by increasing-membrane fluidity, that disorganizes the lipid chains and hinders radical propagation and releases the AST and ALT. Therapeutic applications of various antioxidants have been proved in mammalian models demonstrating restoration of the antioxidant enzyme levels. Compounds protecting from the mitochondrial damage have been tested in mouse and rat models; however, screening for novel compounds which are effective in recovery from acetaminophen induced liver injury is still a necessity. Hepatoprotectants that recover liver from acetaminophen injury rather than antagonizing the mechanism of toxicity are lacking. Therefore, in this study we investigated the effect of heptamethoxyflavone (HMF) on the acetaminophen induced hepatotoxicity in zebrafish and also studied the levels of apoptotic and proapoptotic gene expression.

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Materials and Methods

Preparation of crude extract of *Sphaeranthus amaranthoides* and also isolation, purification and characterization of the active component heptamethoxyflavone (HMF) has been explained in detail in our previous study.\(^\text{10}\)

Animals

Zebrafish were used as animal model and maintained as per the guideline of the committee on protocols and institutional animal care. Normal fish diet was given to the zebrafish. Embryos were collected from adult zebrafish by usual procedure.

Exposure of drug and screening for chemicals

Adult zebrafishes were also maintained according to the protocol and divided into four groups: control (Group I); acetaminophen (Group II) treated; acetaminophen +heptamethoxyflavone (HMF) (Group III); and acetaminophen+silymarin (Group IV). About 10 mM concentration of acetaminophen for 24 h was used as a dose for hepatotoxicity. Groups III and IV after treatment with 10 mM concentration of acetaminophen for 24 h were transferred into the tanks containing heptamethoxyflavone (HMF) 20 µg/L and 20 µg/L of silymarin, respectively. Adult zebrafish were exposed to acetaminophen, isolated flavonoid (HMF) and normal diet at doses as described above. Fertilized eggs were collected from the adult fish of four different groups (from Gr. III and IV after treating with HMF and silymarin) and the developing embryos were maintained at 28°C. Developmental abnormalities and mortality rate was observed every day and recorded under inverted fluorescent microscope.

Biochemical analysis

For biochemical analysis, blood was collected from fish which were anaesthetized in ice chips before blood collection. Then diagonal incision was made with a steel blade just between the anal fin and the caudal fin. Blood was collected using micropipette and 10 fishes blood was pooled to make up to 2 µL and centrifuged the serum were collected. The amount of collected serum was sufficient to analyze the various biochemical parameters.

Assay of Liver marker & antioxidant enzymes analysis and estimation of blood glucose

Levels of ALT, AST measured using spectrophotometer (Cary, varian) and enzymatic kits were used for assay according to the manufacturer instructions. Serum was analyzed for measuring SOD, CAT and GST and determined according to the commercial kits instructions.

For estimation of blood glucose blood was collected and centrifuged following the same procedure as mentioned above. The amount of collected serum was sufficient to analyze the blood glucose.

q-PCR

The q-PCR was performed according to the Raquel Espin-Palazon.\(^\text{2014}\). RNA was isolated from tissues using Rnase. The conditions used to synthesize the cDNA are reverse transcriptase used M-MLV (Promega, Madison, WI) and oligo (dT) primers (Promega) after which q-PCR was performed with cDNAs and gene specific primer pairs (Table 1) and mixed with ABI SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Real-time PCR cycle parameters included 10 min at 95°C followed by 40 cycles involving denaturation at 50°C for 2 min, annealing at 95°C for 10 min 15 s, and elongation at 60°C for 1 min. Detection of Bel2, Bax, caspase 3, and TNF-α was according to manufacturer’s protocols (Sigma) in iQ5 Multicolour RT-PCR detection system (BioRad). Each primer was designed and determined by specificity and linearity of amplification and its melting curve analysis and according to the value of the slope from serial diluted samples (Table 1). Relative fold expression levels of genes were calculated by the following formula:

\[ \text{Relative expression} = 2^{(\text{Ct [sample gene]} - \text{Ct [housekeeping gene]})} \]

Histopathology

Adult zebrafish (6/treatment/2 replicates) liver was collected, sectioned and embedded with PFA paraffin, then stained with H&E and observed under Light microscope.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Bel2</td>
<td>5'-CCAACCTGACAGGAGGC-3'</td>
<td>5'-GATGGTCACTCCGTGCAAGG-3'</td>
</tr>
<tr>
<td>Bax</td>
<td>5'-AAACGCCTGCGGCACTGTG-3'</td>
<td>5'-TCATTGCACTCGCACTGTG-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-CTGAAACAAGCAGCGTTG-3'</td>
<td>5'-CAGCCGCACTTCTGCAAGC-3'</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>5'-TGAGTCACACCTCAACATGCCG-3'</td>
<td>5'-CCGGGAATCCACTTCAACGACC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CGGCGGAGGGAACAACTCTGAA-3'</td>
<td>5'-CATCTCCGTTGACGCTTTC-3'</td>
</tr>
</tbody>
</table>
Statistical analysis

Data was expressed as mean ± S.E.M and analyzed by Turkey’s test to determine the significance of differences between groups. The $P$ value lower than 0.05, 0.01 or/and 0.001 was considered to be significant.

Results and Discussion

Effect of acetaminophen on mortality of zebra fish embryos

Heptamethoxyflavone enhanced the survival rate of zebrafish larvae collected from acetaminophen treated group. Table 2 indicates the effect of HMF on the mortality of embryos. The embryos collected from acetaminophen treated group induced apoptosis and suppressed the embryos growth (Fig. 1). Various parameters were observed for the growth and developmental stages of embryos. Mortality rate was more in acetaminophen treated group, whereas in other groups treated with HMF and silymarin, the mortality rate was less and the embryo development was normal (Fig. 1).

Acetaminophen induced liver toxicity in adult zebrafish

Hepatic marker enzymes

In adult zebrafish, ALT and AST levels were found elevated in the acetaminophen treated group and significant decrease in the group treated with HMF (Fig. 2A). The levels of ALT and AST, markers of hepatic damage clinically, were much more in zebrafish as reported by others in rats, mice and human. The studies on toxicity thresholds were established by recording the survival rate when exposed to higher doses of acetaminophen and proved that 10 mM of acetaminophen causes a progressive death in ~50% of zebrafish\(^\text{11}\). The levels of ALT and AST were significantly normalized after treating with heptamethoxyflavone and silymarin simultaneously when compared with acetaminophen treated group.

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Parameters} & \text{Control} & \text{APAP} & \text{APAP+heptamethoxyflavone} & \text{APAP+Silymarin} \\
\hline
\text{Division} & Normal & Slow & Slow & Slow \\
\text{Growth} & Normal & Slow & Slow/normal & Slow/normal \\
\text{Hatching rate} & 16/20 & 10/20 & 14/20 & 15/20 \\
\text{Swimming behaviour} & Normal & Fast & Slow & Slow \\
\text{Development of liver} & Normal & Damaged & Improved morphology & Improved morphology \\
\text{Body shape} & Straight & Curved & Curved/normal & Straight \\
\text{Tail movement} & 20/min & 12/min & 14/min & 15/min \\
\text{Mortality} & 2/16 & 10/10(after 3 days) & 4/14 & 3/15 \\
\hline
\end{array}
\]

Fig. 1 — Developmental stages of zebra fish embryos isolated from toxicity induced and HMF treated fish

![Fig. 1 — Developmental stages of zebra fish embryos isolated from toxicity induced and HMF treated fish](image)
All these results suggest the zebrafish to be a model for human physiology\textsuperscript{12}.

**Antioxidant enzymes**

In humans, exposure to acetaminophen leads to drastic reduction in antioxidant enzymes such as SOD, CAT and GST in liver, likewise in adult zebrafish 75\% significant reduction of the antioxidant enzyme levels for 24 h post exposures to acetaminophen. Survival of zebrafish embryos and levels of antioxidant enzymes after exposing to flavones were remarkably improved when compared with acetaminophen treated group (Fig. 2B).

**qPCR**

The current study proved that the heptamethoxyflavones treatment against the acetaminophen induced hepatotoxicity could protect from the state of oxidative stress induced. The levels of expression of mRNA of proapoptotic genes and anti apoptotic genes (Bcl-2, bax, caspase-3 and TNF-\(\alpha\)) were studied (Fig. 3). The marked upregulation of antiapoptotic gene (Bcl-2) expression has been observed in Gr. III (heptamethoxyflavone treated) by indicating its protective nature against the acetaminophen toxicity. Whereas in the acetaminophen treated group there is no change in the levels of Bcl-2 expression. The expression of the apoptotic genes like caspase-3 and TNF-\(\alpha\) expression was downregulated in silymarin treated and flavones treated groups. GeneEX software was used to calculate the Ct values of q-PCR data and normalized with GAPDH.

**Histopathology**

The histopathological evaluation using H & E staining revealed the acetaminophen treated group showing hepatic necrosis, sinusoidal widening and there are wide spread areas of focal haemorrhage. Fig. 4A showed normal architecture with large nucleus and granular cytoplasm without any infiltration of cells. The acetaminophen and post flavone treated group showed marked reduction in the necrosis and haemorrhage (Fig. 4 B and C). Reduction in the necrosis indicated during H & E staining and improvement in the liver morphology in flavones post treated group correlated with levels of hepatic marker enzymes and antioxidant enzymes this was significant when compared with sylimarin (Fig. 4D). The histology of liver from HMF treated group improved liver condition and showed very less portal vein congestion and leucocytic infiltration with no vaculation of periportal hepatocytes.

In the present study, we identified that heptamethoxyflavone plays a critical role in protecting liver during the toxicity induced by acetaminophen. Hence, here we find out the hepatoprotective activity of heptamethoxyflavone in zebrafish embryos and adults against the paracetamol induced hepatotoxicity. The hepatotoxicity with paracetamol is due to the formation of reactive toxic electrophile intermediate, NAPQI by cytochrome P-450\textsuperscript{13}. Antioxidant enzymes such as reduce glutathione can conjugate to NAPQI and detoxify their toxicity, however the increase in NAPQI raise depletes the GSH. These results in upregulation of mRNA for antiapoptotic gene (bcl-2) in Gr. III and IV. But in Gr. II (paracetamol alone treated) the expression levels of mRNA for bcl-2 gene was significantly reduced when compared to the flavones treated group.
Flavone treatment significantly prevented the expression of bax, caspase and TNF-α when compared with the acetaminophen treated group. The molecular mechanism of toxicity of acetaminophen was elucidated in various in vitro and in vivo models. The previous strategies of treatments are on the principle of the GST replenishing and upregulation of antiapoptotic genes and downregulation of proapoptotic genes. Zebrafish has been a model for hepatotoxicity and also for drug screening for their physiological relevance. The in vivo animal models are helpful in assessing the human liver toxicity and depend mostly on valid assays to determine toxin intermediate effects. According to the previous studies, acetaminophen significantly lowers hepatic GST, SOD and CAT, and their levels improve with heptamethoxyflavone. These observations are in alignment with our results in this study as well. ALT and AST levels are standard liver functional tests in human clinical practice, which were also quantified here to estimate the liver specific toxicity. Histopathological parameters, such as hepatocyte necrosis and viability also play an important role with clinical experience.

Zebrafish have applicability in performing the unique featureat in vivo chemical screening in an integral vertebrate organism. There are no prior in vivo studies to explore the potential to extend and enhance the therapeutic effects of heptamethoxyflavone for acetaminophen liver injury. In the current study, we have demonstrated the significance of heptamethoxyflavone in protecting both embryos and adult fishes against acetaminophen induced hepatotoxicity. Earlier, we have demonstrated the hepatoprotective activity of crude extract of Sphaeranthus amaranthoides in rat model. The therapeutic effect of heptamethoxyflavone administration against acetaminophen liver injury, could be attributed to the modulation of various mechanisms of liver damage, viz. restoration of antioxidant enzymes and maintenance of hepatic marker enzymes levels. The restoration of antioxidant enzymes mediated by heptamethoxyflavone combined with a proliferative, antiapoptotic signal from Bcl2; unlikely, when treated with heptamethoxyflavone, acts as antidote to the potential cyclooxygenase inhibition induced by acetaminophen.

Maintenance of markers of liver injury in zebrafish could lead to more common applications of this model to evaluate drug induced liver injury. Incidence of hepatotoxicity, as a major side effect, is a serious problem for many therapeutic molecules in development, preventing them from attaining delayed clinical trial stages.

Conclusion

Heptamethoxyflavone, a polyphenol acts as an activator possessing an antioxidant role by enhancing the hepatoprotective nature. The current in vivo study proved that heptamethoxyflavone (HMF) can be a potential drug to protect against the toxicity induced by the acetaminophen. The significant reduced levels of liver marker enzymes conclude that heptamethoxyflavone has a potential role in maintaining the levels and the activity of liver marker enzymes and antioxidant enzymes.

Reference

3. Craig DG, Bates CM, Davidson JS, Martin KG, Hayes PC & Simpson KJ, Staggered overdose pattern and delay to


