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Effects of acetaminophen administration on liver histopathology, serum GOT/GPT levels and circulating microRNA-122 concentration in olive flounder (*Paralichthys olivaceus*)

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In human medicine, circulating microRNAs have been successfully utilized as early biomarkers for various abnormalities and disease states. Vertebrate miR-122 is a liver-specific microRNA which is expressed almost solely in hepatocytes and plays an important role in the regulation of hepatocyte function. In this study, to evaluate the potential utility of circulating miR-122 as a biomarker for liver injury in olive flounder (*Paralichthys olivaceus*), fish were orally intubated with two doses of acetaminophen (500 mg/kg or 1.000 mg/kg of body weight), and the expression of miR-122 in serum was quantified using real time-PCR. Histological change in liver, and the enzymatic activity of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were also analyzed. The results showed that miR-122 was higher in acetaminophen administered groups compared to control group. The histopathological effect of acetaminophen on olive flounder liver was not distinct. The serum level of GPT and GOT was increased within 2 folds compared to control group by acetaminophen administration. However, the serum miR-122 level was increased more than 3 or 4 folds compared to the control group by administration of 1000 mg/kg of acetaminophen. These results suggest the possible use of miR-122 as an indicator of liver injury in olive flounder, even when histopathological effects are not distinctive.

Key words: Circulating microRNA-122, Acetaminophen, Olive flounder, Liver toxicity, Indicator

MicroRNAs are small, noncoding RNAs, involved in post-transcriptional regulation of mRNA through binding to the untranslated sequence of mRNA inducing gene silencing. Furthermore, each microRNA have several target genes, and the individual target gene can be under the control of different microRNAs involved in various biological process, which allows a more complex role of microRNAs in regulation of biological processes (Bartel, 2004). This complexity and flexibility allow to microRNAs to play a pivot role in several biological processes such as development (Carrington, Ambros, 2003), immunity (Teleman, Cohen, 2006) and apoptosis (Ding, Voinnet, 2007; Lima et al., 2011).

MicroRNAs are first transcribed as primary micro RNAs (pri-microRNAs) in the nucleus and then generate around 70 nucleotides precursor microRNAs (pre-microRNAs) having a stem loops through processing by Drosha. The pre-microRNAs are transported to the cytoplasm to be trimmed by the Dicer into RNA duplex with 20 bp in length and 2-nt

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3'overhangs. Furthermore, the mature microRNAs will be integrated into the RNA induced silencing complex (RISC), and the formation of duplex between microRNA-mRNA will leads to inhibition of translation (Cai, Hagedorn, Cullen, 2004; Hutvágner, Zamore, 2002).

MicroRNAs are not limited to the cell, but also can be secreted into the body fluids, called circulating microRNAs. The presence of ubiquitous ribonucleases (RNases) in circulation can lead to degradation of circulating microRNAs, but the secretion of microRNAs into circulation is done by packing them into microparticles (exsosomes, microvesicles, and apoptic bodies) or by linking them to RNA-binding proteins such as Argonaute 2 or lipoprotein complexes (Chim et al., 2008; Gibbings, Ciaudo, Erhardt, Voinnet, 2009; Valadi et al., 2007; Zernecke et al., 2009).

In human medicine, the circulating microRNAs are already been used as early biomarkers for several diseases, such as esophageal squamous cell carcinoma detection (C. Zhang et al., 2010), identifying Parkinson's disease onset and disease progression Margis, (Margis, Rieder, 2011), and diagnosis of hepatocarcinoma (Budhu et al., 2008) etc., proving that micro RNAs could be an new area to find specific and early biomarkers for different diseases.

Several reports confirmed the liver specificity of miR-122 which have a high expression in hepatocytes over 50.000 copies per cell (Filipowicz, Großhans, 2011). MiR-122 plays crucial role in the hepatocytes differentiation, liver development (Xu et al., 2010), and lipid metabolism (Filipowicz, Großhans, 2011). Since the miR-122 is located and highly expressed in liver, divers studies focused on importance of circulating miR-122 as potential early biomarker for liver injury caused by drugs (Laterza et al., 2009; Starkey Lewis et al., 2011; Wang et al., 2009; Y. Zhang et al., 2010).

The acetaminophen (paracetamol) is the most widely used drug in human medicine as analgesic without a prescription, however overdosing can lead to an acute liver failure (Bower, Johns, Margolis, Williams, Bell, 2007). High doses of acetaminophen causes destruction of liver normal architect through centrilobular hepatic necrosis (Ramachandran, Kakar, 2009). The major factor causing the hepatotoxicity of acetaminophen is a highly reactive N-acetyl-p-benzoquinone-imine (NAPQI) which is produced as an intermediate metabolite from acetaminophen through CYP2E1, CYP3A4, and CYP1A2 located in the centrilobular regions of liver (Moyer et al., 2011). Normally, the NAPQI is eliminated through conjugation with glutathione (GSH). Yet, in case of high administration of acetaminophen, this mechanism is overloaded and induce formation of binding between the NAPQI and hepatocytes proteins (Cohen et al., 1997). In mammals, divers studies showed the correlation between high level of miR-122in plasma or serum and the overdosed mice with acetaminophen or drug-induced liver injury for human (Antoine et al., 2013; Starkey Lewis et al., 2011; Thulin et al., 2014; Wang et al., 2009). Furthermore, the miR-122 is highly abundant in the liver (Chang et al., 2004), which makes miR-122 an excellent choice to be a candidate for early biomarker in liver.

Recently, although several studies focused on the microRNAs in aquatic animals particularly in fish (Campos et al., 2014; Ma, Hostuttler, Wei, Rexroad,Yao, 2012; Xiao et al., 2014), as far as we know, there is only one paper that investigated on the circulating microRNAs in fish, in which Vliegenthart et al. (2014) reported that zebrafish (*Danio rerio*) exposed to 20-40 mM acetaminophen-dissolved water for 3 h showed hepatotoxicity-mediated histological changes and a high expression of miR-122 in serum. Olive flounder (*Paralichthys olivaceus*) is the major marine cultured fish in Korea, and expression of microRNAs in the tissues of olive flounder has been reported (Fu et al., 2011; Xie et al., 2011). In this study, we have investigated on the hepatotoxicity of an oral over-dose of acetaminophen in olive flounder and evaluated the potential utility of circulating

miR-122 as a marker of liver injury in olive flounder.

Material and Methods

Fish and experimental design

Forty five individuals of olive flounder weighing approximately 100 g were obtained from a local farm, and placed in three separate 500 l tanks (15 fish in each tank). After being acclimatized for one week, the control group was orally administrated 100 μl of phosphate buffered saline (PBS). The treatment groups were administrated acetaminophen (Sigma) per os at a dose of 500 mg/kg and 1000 mg/kg, respectively, using intubation tubes. Blood samples and liver samples were collected at 12, 24, and 48 h post-administration of acetaminophen from randomly sampled 5 fish in each group.

Serum GOT and GPT analysis

The blood collected from the caudal vein was kept at room temperature for one hour, centrifuged at 8000 rpm for 10 min at 4°C, and stored at -80°C. Levels of GOT and GPT were detected using ASAN kit (Asan Pharm., Korea) based on Reitman-Frankel method (Reitman, Frankel, 1957).

Histological and pathological assay

The liver was placed in Bouin solution and left to fix for at least 24 h, and embedded in paraffin. Tissue sections were prepared with a microtome at 5 μm and placed on glass slides. Liver lesions were observed microscopically after hematoxylin and eosin (HE) staining.

RNA isolation from serum

Total RNA, including small RNAs, were isolated from 100 μl of serum using the Qiazol extraction method followed by column purification with a miRNeasy serum Mini kit (Qiagen) in accordance with the manufacturer's protocol. Briefly, 500 μl of Qiazol and 100 μl of chloroform were added to 100 μl of serum, followed by centrifugation for 15 min at 12,000 g at 4°C. Next, 300 μl of the RNA-containing aqueous phase was transferred into a new tube, and RNA was precipitated with 450 μl of 100% ethanol and loaded on miRNeasy purification columns. Purified RNA was eluted from the column matrix with 14 μl of RNase free water. To control extraction efficiency and variability, one synthetic *Caenorhabditis elegans* microRNA, syn-miR-Cel-39, was spiked into each sample during the Qiazol step. To aid in improving the RNA yield, total RNA from bacteriophage MS2 was added to each sample as carrier.

MicroRNA detection and quantification

RNA (2 μl) was reverse-transcribed in 10 μl reactions using miScript II RT Kit (Qiagen). cDNA was diluted 20x and assayed in 20 μl PCR reactions according to the manufacture's protocol for miScript SYBR Green PCR Kit using microRNA-122-specific forward primer (TGGAGTGTGACAATGGTGTTTG) or cel-miR-39 specific forward primer (Qiagen), a control endogenous microRNA, and the universal primer (Qiagen) in Roche light cycler 480. Cycling conditions were 95°C for 15 min followed by 45 cycles of 94°C for 15s and 55°C for 30s. MicroRNA levels were normalized to the levels of the spike in control cel-miR-39 and the relative microRNA production was determined with ΔCt method and reported as $2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle. Differences in microRNA expression in treatment groups compared to control group were expressed as fold change.

Statistics

Statistical analysis was performed using SPSS v 20. Differences among groups were analyzed using ANOVA followed by Tukey HSD post-hoc test. $P< 0.05$ was considered statistically significant.

Results

Liver histopathology

Sections of liver were observed under a light microscope. Most of samples didn't show any significant lesion such as inflammation or necrosis as observed in mammals caused by acetaminophen toxicity on liver. The most fish even in the control fish showed atrophy of hepatocytes with presence of lipid vacuolation in some cases (Fig. 1).

GOT and GPT analysis

Both concentrations of acetaminophen used for inducing liver toxicity brought an increase of GPT activity and showed significant differences compared to the control group at 48 h post-administration (Fig. 2A). Although the GPT activity values of fish administered acetaminophen at a dose of 1,000 mg/kg were continuously higher than fish administered 500 mg/kg of acetaminophen, there were no statistical differences between the two groups (Fig. 2A).

Only the fish administered acetaminophen at a dose of 1,000 mg/kg showed increased activity of GOT. The serum GOT activity of fish administered 1,000 mg/kg of acetaminophen was significantly higher than that of fish administered 500 mg/kg of acetaminophen or fish in the control group at 24 h post-intubation (Fig. 2B). The GOT activity was declined at 48 h post-administration.

Level of miR-122 in serum

The oral administration of acetaminophen resulted in a dose-dependent increase of miR-122 in serum, and reached its peak at 24 h post-administration of acetaminophen (Fig. 3). The fish administered 1,000 mg/kg of acetaminophen at 48 h post-intubation showed significant increase of serum miR-122 compared to fish in the control group (Fig. 3).

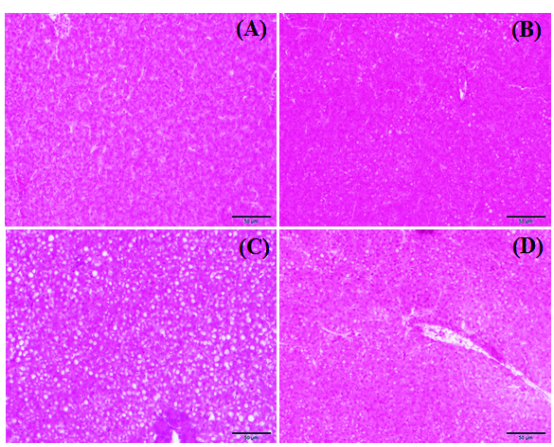

Fig. 1. Effect of acetaminophen on the histology of liver. Hematoxylin and eosin staining of liver sections of olive flounder (*Paralichthys olivaceus*). (A) Control group at 48 h post oral administration of 100 µl of phosphate buffered saline (PBS), (B) Treatment group at 6 h post oral administeration of 500 mg/kg of acetaminophen, (C) Treatment group at 24 h post-administration of 1000 mg/kg of acetaminophen, (D) Treatment group at 48 h post-administration of 1000 mg/kg of acetaminophen.

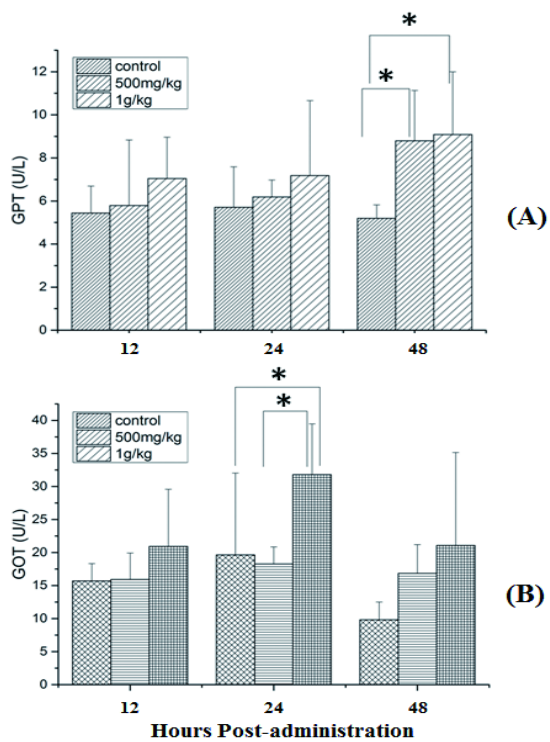

Fig. 2. Effect of acetaminophen on GPT (A) and GOT (B) levels in the serum of olive flounder (*Paralichthys olivaceus*). Values are mean + standard deviation. Asterisks represent statistically significant at $P<0.05$ between groups connected by an inflexed line.

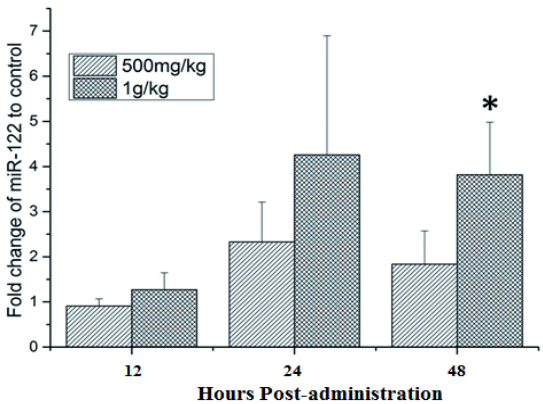

Fig. 3. Effect of acetaminophen on the concentration of miR-122 in the serum of olive flounder (*Paralichthys olivaceus*). Fold change of serum miR-122 in fish orally administered acetaminophen at a dose of 500 mg/kg or 1000 mg/kg compared to that in control group. Values are mean + standard deviation. The asterisk on the bar represents statistically significant at $P<0.05$.

Discussion

In the present study, we have investigated the effect of acetaminophen on liver histopathology, serum GPT and GOT activities, and the level of circulating microRNA-122. In mammals, acetaminophen produced centrilobular necrosis and congestion in the liver of rat and mice (Blazka, Elwell, Holladay, Wilson, Luster, 1996; Gardner et al., 1998; Laskin, Gardner, Price,Jollow, 1995; Lee et al., 2001). However, in this results, the histopathological effect of oral acetaminophen administration on olive flounder liver was not distinctive. Previous reports also showed that histopathological effect of acetaminophen in fish administered oral or bath route at a dose of 500 mg/kg was not severe compared to mammals (Blair, Hinton, Miller, 1989; Kavitha, Ramesh, Bupesh, Stalin, Subramanian, 2011; Shivashri, Rajarajeshwari, Rajasekar, 2013). Although Vliegenthart et al. (2014) reported hepatocytes necrosis in zebrafish by bath exposure to acetaminophen, very high concentrations of acetaminophen (20-40 mM; about 3000-6000 mg/L) used in the study might be the cause of the severe hepatotoxicity. In mammals, the electrophilic intermediate NAPQI causes a depletion of its detoxificant, glutathione, causing a periportal necrosis (Gabriel, Michel, 2007; Klaassen, 2008). However, in fish, the relative low ability to produce NAPQI from acetaminophen was suggested as a cause of the reduced hepatotoxic effect of acetaminophen (Hinton DE, 2001; Thomas, Wofford, 1984). Most of fish used in this study including control fish showed atrophy accompanied sometimes with lipid degeneration in the liver, which might shade the effect of acetaminophen on histological change of the liver.

The GOT and GPT are enzyme of mitochondrial localization involved in the metabolism of amino-acids (Gharaei, Ghaffari, Keyvanshokooh, Akrami, 2011) and principally find in higher concentration in liver, muscle, kidney and heart. High level of GOT and GPT in blood can be an indicator of leakage from cells caused by damage of cell membrane. Hence, the evaluation of this two enzymes in blood is a routine procedure to evaluate and monitor the hepatocellular disorders or muscle damage (Asha, Akhila, Wills, Subramoniam, 2004; Yanpallewar et al., 2003; Yen, Wu, Lin, Lin, 2007). In the present results, the activities of GOT and GPT were increased by oral administration of acetaminophen, however the increased amplitude was much lower than in rodents administered a similar dose (Oliveira et al., 2005) or even a smaller dose (Knight and Jaeschke, 2002). This relatively weaker effect of acetaminophen on GOT and GPT of olive flounder may be explained by the relative inability of hepatocytes to transform acetaminophen to its reactive toxic intermediate (Blair et al., 1989; Thomas, Wofford, 1984).

In mammals, even though that liver is rich in GOT and GPT enzymes, any increase in serum of GPT activity is not an indicator of liver injury, but can arise from extra-hepatic injury, particularly as response to skeletal muscle injury in response to inflammation, overuse, or traumatic conditions (Nathwani, Pais, Reynolds, Kaplowitz, 2005). Similar findings were

published in fish (Anderson, Stoskopf, Morris, Clarke, Harms, 2010; Samanta, Pal, Mukherjee, Ghosh, 2014), which indicates that liver enzymes (GPT and GOT) are less specific biomarker to be used in liver injury. MicroRNAs inhibit translation of mRNAs and play a pivot role in regulation of divers physiological and pathological processes (Carthew, Sontheimer, 2009). Moreover, circulating microRNAs became an important area of study in human medicine for their use as biomarkers of various pathologic mechanisms (Margis et al., 2011; Pineau et al., 2010; C. Zhang et al., 2010). In this study, the quantity of circulating miR-122 was dose-dependently elevated by oral administration of acetaminophen. As the liver is the richest organ for miR-122, increased amount of miR-122 in the blood might suggest an injury in the liver, which were verified in previous studies with zebrafish (Vliegenthart et al., 2014) and mammals (Laterza et al., 2009; Y. Zhang et al., 2010).

In this study, the histopathological effect of acetaminophen on olive flounder liver was not distinct. The serum levels of GPT and GOT were increased within 2 folds compared to control group by acetaminophen administration. However, the serum miR-122 level was increased more than 3 or 4 folds compared to the control group by administration of 1000 mg/kg of acetaminophen. These results suggest that circulating miR-122 might be used as a sensitive indicator as well as GOT and GOT levels in evaluating liver injury in olive flounder, even when histopathological effects are not distinctive.

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