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Circulating Plasma and Exosomal microRNAs as Indicators of Drug-Induced Organ Injury in Rodent Models

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Abstract

This study was performed to evaluate whether microRNAs (miRNAs) in circulating exosomes may serve as biomarkers of drug-induced liver, kidney, or muscle-injury. Quantitative PCR analyses were performed to measure the amounts of liver-specific miRNAs (miR-122, miR-192, and miR-155), kidney-specific miR-146a, or muscle-specific miR-206 in plasma and exosomes from mice treated with liver, kidney or muscle toxicants. The levels of liver-specific miRNAs in circulating plasma and exosomes were elevated in acetaminophen-induced liver injury and returned to basal levels by treatment with antioxidant *N*-acetyl-cysteine. Circulating miR-146a and miR-206 were increased in cisplatin-induced nephrotoxicity and bupivacaine-induced myotoxicity, respectively. Taken together, these results indicate that circulating plasma and exosomal miRNAs can be used as potential biomarkers specific for drug-induced liver, kidney or muscle injury.

Key Words: miRNAs, Exosomes, Liver-specific injury, *N*-acetyl cysteine, Biomarkers

INTRODUCTION

MicroRNAs (miRNAs) play important roles in regulating many cellular processes in normal physiological and pathological conditions. Circulating miRNAs have been recently suggested to serve as easily accessible biomarkers for diagnosis of cancer and other disease states (Yu *et al*., 2011; Guay and Regazzi, 2013). More importantly, high levels of circulating miRNAs are produced within certain cells in a tissue-specific manner (Lagos-Quintana *et al*., 2002; Ason *et al*., 2006). One of the most recent exciting findings is that miRNAs exist in exosomes. Exosomal miRNAs can be physically transferred to target cells and play an important regulatory role in diverse biological processes. In addition, miRNAs in exosomes from blood samples have been shown to be stable even under extreme conditions, making them excellent candidates for non-invasive biomarkers (Turchinovich *et al*., 2011).

Previous studies have considered the potential use of miRNA species as biomarkers in acetaminophen (APAP)-induced liver injury. For instance, in the animal models of liver injury, circulating miR-122, miR-192, and miR-155 may reflect liver damage and inflammation (Wang *et al*., 2009; Starkey Lewis *et al*., 2011; Bala *et al*., 2012). In addition, circulating miR-122 was confirmed as a sensitive and reliable blood marker for drug-, viral-, alcohol-, and chemical-induced liver injury (Zhang *et al*., 2010). John *et al*. (2014) also reported that the levels of miR-122 in serum and liver tissues were elevated in acute liver failure patients. The levels of circulating miR-146a, which is considered kidney-specific due to its high expression in the kidney, has been shown to increase following chronic kidney disease in mice and humans (Wang *et al*., 2011; Ichii *et al*., 2012). The miR-206 is specifically expressed in skeletal muscle (Sempere *et al*., 2004) and released into the plasma in the muscle-related disorders (Mizuno *et al*., 2011; Toivonen *et al*., 2014). Although liver-specific circulating miRNAs in drug-induced liver injury have been recently reported, circulating plasma and exosomal miRNAs in injury of other organs have not well-established. Therefore, we evaluated whether circulating miRNAs in plasma and exosomes can serve as easily accessible biomarkers of drug-induced organ injury. In this study, we specifically focused on the levels of candidate miRNAs such as miR-122, miR-155, and miR-192 (as liver specific), miR-146a (as kidney specific), and miR-206 (as muscle specific) after treatment with an organ-specific toxicant. Our

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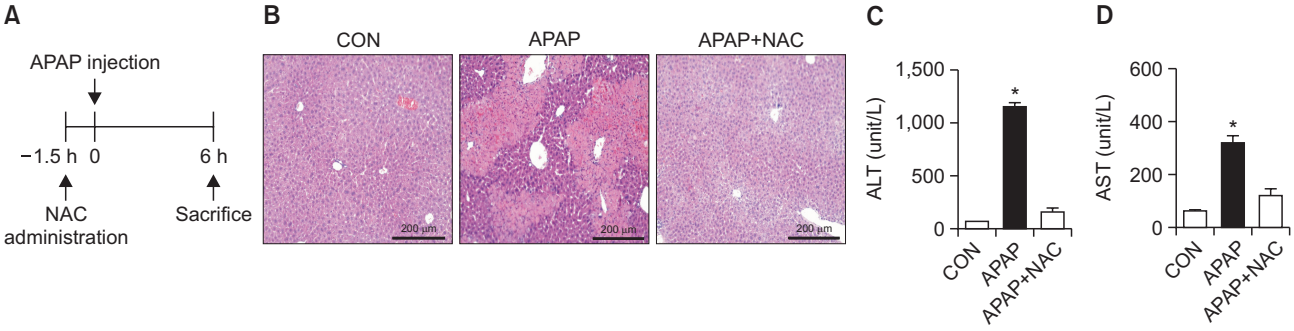

Fig. 1. Confirmation of APAP-induced liver injury. (A) NAC pretreatment protocol in a mouse model of APAP-induced liver injury. (B) Representative images of hematoxylin and eosin (H&E) staining for formalin-fixed liver sections in mice pretreated with or without NAC. Scale bars, 200 μm. (C, D) Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (n=5/group). The data represent mean ± SD, **p*<0.05.

results show that increased levels of circulating miR-122, miR-155, and miR-192 correlate with the degree of liver injury, while circulating miR-146a and miR-206 correlate with kidney and muscle injury, respectively. Furthermore, we showed for the first time that the identities of exosomal miRNAs could reflect drug-induced organ injury.

MATERIALS AND METHODS

Animals studies

The animal studies were approved by the Institutional Animal Use and Care Committee of the Kyungpook National University. Male 6-week old Balb/C mice (n=5/group) were fasted overnight before they were treated with a single i.p injection with PBS (phosphate buffered saline) as control (CON), APAP (300 mg/kg, Sigma, St. Louis, MO, USA), or cisplatin (CIS, 10 mg/kg, Sigma) for 24 h. In addition, bupivacaine-HCl (BPVC, 0.4 mL of 0.5% wt/vol, Sigma) dissolved in PBS, was injected once into both the right and left tibialis anterior of the mice.

For protection against APAP-induced liver injury, antioxidant N-acetyl cysteine (NAC, 100 mg/kg, Sigma) was administrated i.p. 1.5 h prior to APAP injection. To protect against CIS-induced kidney injury, mice were treated with quercetin (QR, 100 mg/kg/day), which was administrated orally for 10 consecutive days after mice were exposed to a single i.p injection with cisplatin (10 mg/kg).

ALT and AST analysis

ALT and AST levels were determined in plasma obtained from the individual animals by using a standard end-point colorimetric assay kit (TECO Diagnostics, Anaheim, CA, USA).

Histological analysis

Formalin-fixed liver, kidney, and muscle tissues were stained with hematoxylin-eosin and examined with a light microscope (Nikon, Tokyo, Japan; original magnification, ×10 to ×20).

Exosomes isolation

Plasma was mixed with ExoQuick exosome precipitation solution to isolate exosomes by following the manufacturer's protocol (SBI System Biosciences, Palo Alto, CA, USA). Briefly, after incubation at 4°C for 30 min, the mixed samples were centrifuged at 1,500×g for 30 min. The exosome fraction was washed twice with PBS and lysed with QIAzole (Qiagen, Valencia, CA, USA) and RIPA buffer for isolating total RNA and protein, respectively.

miRNA extraction from plasma and exosomes

The miRNA was extracted using a miRNeasy kit (Qiagen) by following the manufacturer's instructions with minor modifications. Synthetic Caenorhabditis elegans (cel)-miR-39 was spiked before miRNAs were further purified by using the miRNeasy kit protocol (Qiagen). The quality and quantity of the RNA containing miRNA components from plasma or exosomes were evaluated by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Real-time quantitative RT-PCR analysis

The expression levels of miRNA were confirmed with SYBR-based quantitative PCR (qRT-PCR) using individual miRNA-specific primers (Qiagen). The first-standard miRNA-cDNA PCR template was generated from total RNA according to the manufacturer's instructions. Approximately 2.5 ng of cDNA was then used in the PCR. The level of specific miRNA based on SYBR green intensity was then monitored with the ABI7500HT real-time PCR system (Applied Biosystems, Hercules, FL, USA). Cel-miR-39 was used to normalize the technical variation between the samples.

Transmission electron microscopy

For negative staining, isolated exosomes were fixed in 2.5% glutaraldehyde (vol/vol) in cacodylate buffer. Exosomes were adsorbed onto 400 mesh carbon-coated copper grids and stained with 0.75% uranyl formate (wt/vol). Samples were observed under a FEI Tecnai G2 Spirit transmission electron microscope (North America NanoPort, OR, USA) operated at a 60 kV accelerating voltage. Images were recorded with an Olympus SIS Veleta CCD camera (Olympus, Center Valley, PA, USA).

Western blot analysis

Exosomal preparations were lysed with RIPA buffer to isolate exosomal proteins. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo, MA, USA), and equal amounts of protein from each sample were separated by SDS/PAGE for immunoblot analyses, as describe (Cho *et al*., 2012).

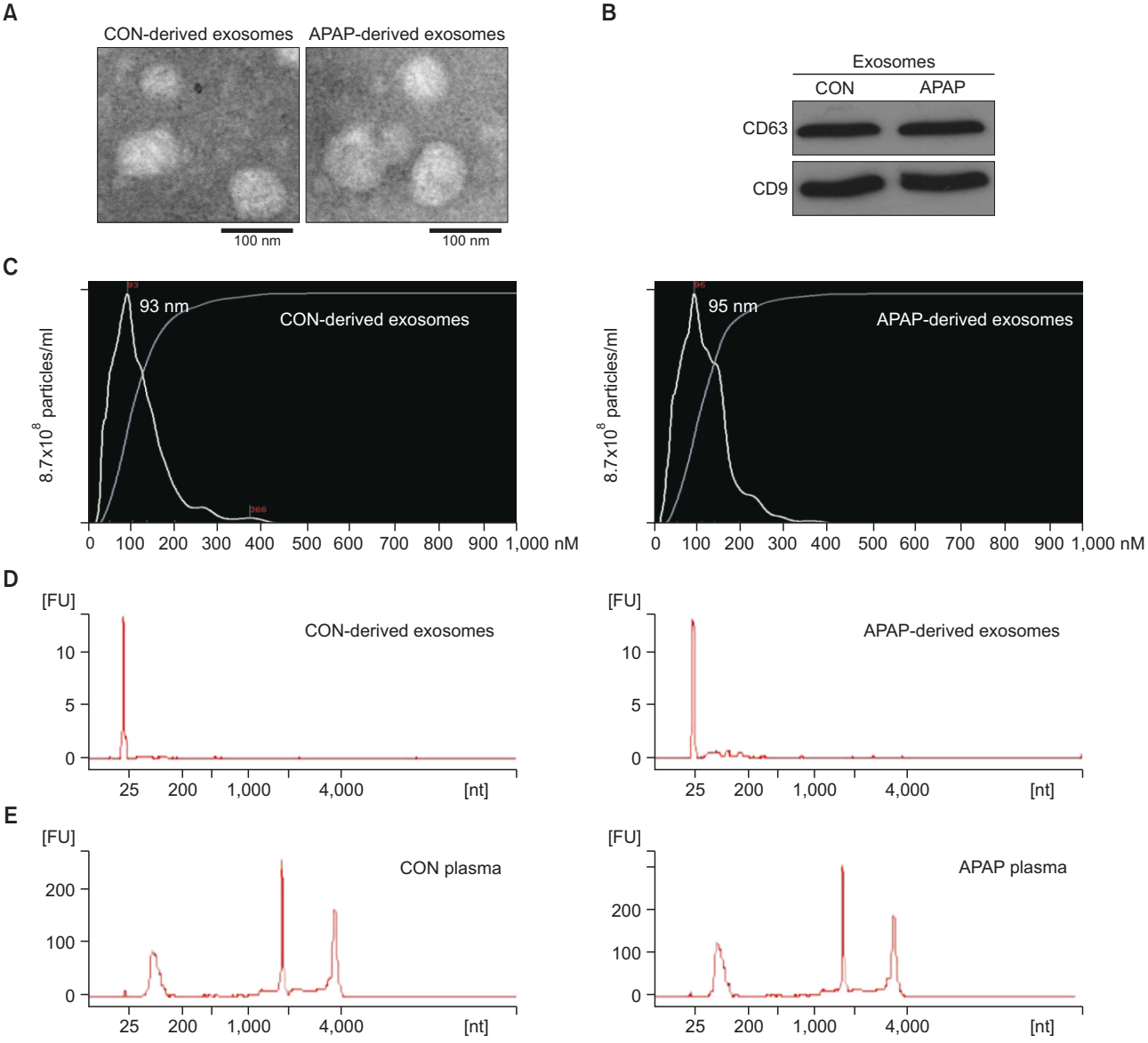

Fig. 2. Characterization of exosomes isolated from individual mouse plasma. (A) A representative electron microscopic image of EVs isolated from the individual mouse plasma from PBS (CON) or APAP-exposed group. Scale bar, 100 nm. (B) Representative immunoblot images of exosome markers (i.e., CD63 and CD9). (C) Analysis of the size distribution of EVs from CON- or APAP-exposed mice. (D) miRNA content of exosomes from PBS (CON) or APAP-exposed mice analyzed by capillary electrophoresis. (E) miRNA content of plasma from PBS (CON) or APAP-exposed mice analyzed by capillary electrophoresis.

Statistical analysis

Data are presented as mean ± SD as indicated for each graph. Means and standard deviations were calculated using SPSS 17.0 (IBM Inc., Brea, CA, USA). Student's *t*-test was used to evaluate differences in means for normally distributed data. All *p*-values are two-tailed, and values of less than 0.05 were considered to indicate statistical significance. **p*<0.05.

RESULTS

Hepatotoxicity induced by acetaminophen

APAP overdose is the predominant cause of acute drug-induced liver injury (DILI) and leads to mitochondrial dysfunction and nuclear DNA fragmentation, resulting in necrotic liver cell death (Hinson *et al*., 2010). We used a protocol of NAC-mediated protection against APAP-induced acute liver injury, as shown in Fig. 1A. The histological examination of liver tissues confirmed APAP-induced liver injury with the classical centrilobular necrosis (Fig. 1B). However, NAC pretreatment fully prevented APAP-induced liver injury (Fig. 1B). APAP exposure markedly increased the serum ALT and AST levels, frequently used as biomarkers for liver injury, compared to the control group (Fig. 1C, 1D). NAC pretreatment significantly suppressed the ALT and AST levels elevated by APAP (Fig. 1C, 1D). These results indicated that our mouse model of APAP-

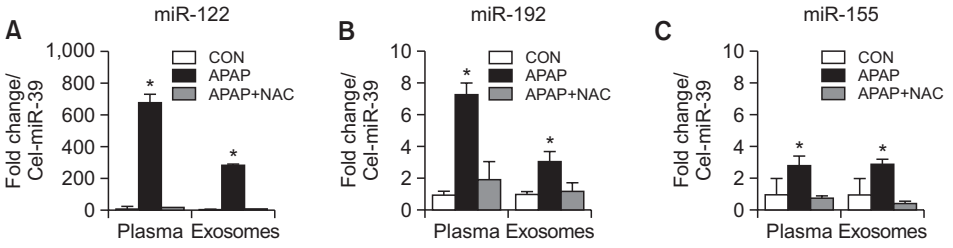

Fig. 3. Increased amounts of miR-122, miR-192, and miR-155 in circulating plasma and exosomes in APAP-mediated liver injury. The amounts of miR-122 (A), miR-192 (B), or miR-155 (C) in plasma and exosomes from CON-, APAP- and APAP+NAC-treated mice were determined (n=5/group). The data represent mean ± SD, **p*<0.05.

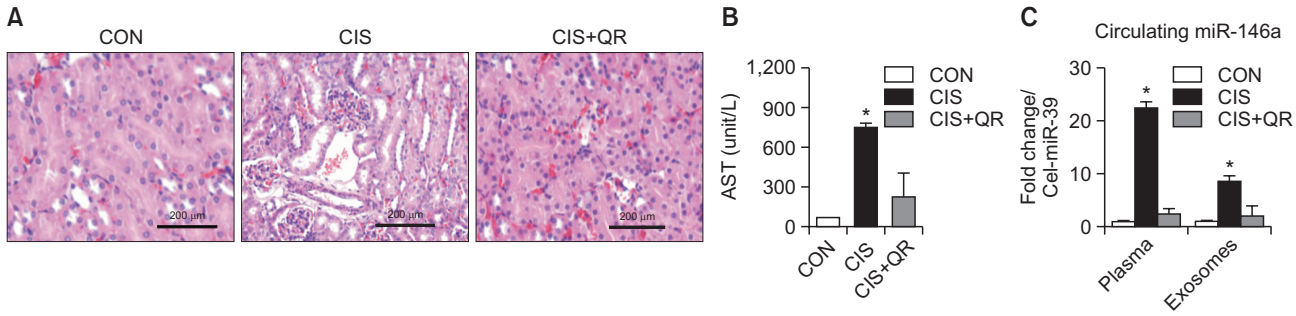

Fig. 4. Increased amounts of miR-146a in circulating plasma and exosomes in CIS-induced kidney injury. (A) Representative images of hematoxylin and eosin (H&E) staining of formalin-fixed kidney sections with or without QR treatment. Scale bars, 200 μm. (B) Plasma AST levels (n=5/group). (C) The amounts of miR-146a were quantified in circulating plasma and exosomes from CON- and CIS- mice with or without quercetin antioxidant (n=5/group). The data represent mean ± SD, **p*<0.05.

induced hepatotoxicity was successfully established.

Characterization of APAP-derived exosomes

To confirm the purity of exosomes, we first examined the size of CON- and APAP-derived exosomes under electron microscopy and validated the size range of less than 100 nm (Fig. 2A). In addition, identification of isolated exosomes was demonstrated by the presence of exosomal biomarkers, CD63 and CD9 (Fig. 2B). Furthermore, Nanoparticle Tracking Analysis (NanoSight) verified the average size of CON- and APAP-derived exosomes 93 and 95 nm, respectively (Fig. 2C).

Elevation of circulating liver-specific miR-122, miR-192, and miR-155 in APAP-induced liver injury

We first evaluated the profile of total RNA isolated from circulating exosomes by capillary electrophoresis. Compared to the plasma, RNA extracted from circulating exosomes did not show clear bands of 18S and 28S rRNA, and the RNA content of circulating exosomes was selectively enhanced for small RNAs, such as miRNAs (Fig. 2D, 2E). We also specifically examined the levels of miR-122, miR-192, and miR-155 expression in circulating exosomes from plasma in mice treated with PBS (CON) or APAP. Our results revealed that APAP administration markedly increased hepatocyte-specific miRNAs (i.e., miR-122 and miR-192) while it modestly elevated inflammatory miRNA (i.e., miR-155) in plasma (Fig. 3). Interestingly, in APAP-induced liver injury associated with massive hepatocyte necrosis, the levels of miR-122, miR-192, and miR-155 in circulating exosomes were significantly increased (Fig. 3). To further validate the utility of hepatocyte-associated miRNAs as potential liver-injury biomarkers, we also studied the effects of NAC pretreatment on the levels of exosomal miRNAs. The elevated levels of the circulating miR-122, miR-192, and miR-155 in plasma and exosomes following APAP-treatment were significantly decreased and returned to basal levels by NAC pretreatment (Fig. 3). These results suggest that miR-122, miR-192, and miR-155 in circulating exosomes mirror hepatocyte damage and thus can be used as potential biomarkers for liver injury.

Elevation of circulating kidney-specific miR-146a in CIS-induced kidney injury

Cisplatin nephrotoxicity is the composite result of the transport of cisplatin into renal epithelial cells, injury to nuclear and mitochondrial DNA, activation of a multiple cell death and inflammation (Miller *et al*., 2010). We evaluated the levels of circulating miR-146a as the target gene of CIS-induced kidney injury because miR-146a is expressed in renal tissues and that its elevated expression in kidney and urines was observed in a murine model of chronic kidney disease (CKD) (Ichii *et al*., 2012). To further validate the utility of miR-146a as a kidney disease-specific biomarker, we also tested the effects of quercetin (QR), which effectively prevented CIS-induced nephrotoxicity through its strong antioxidant and anti-inflammatory properties (Sanchez-Gonzalez *et al*., 2011). Histological analyses revealed that CIS caused serious tubular damage, while co-treatment with quercetin effectively reduced the number of tubular necrosis (Fig. 4A). Quercetin administration significantly prevented the CIS-mediated elevation of the plasma AST level (Fig. 4B). In addition, circulating plasma and exosomal miR-146a levels, elevated in CIS-induced kidney injury, were restored by quercetin administration (Fig. 4C). Thus, antioxidant quercetin sufficiently blocked some of cisplatin-mediated kidney injury and the elevated levels of circulating exosomal and plasma miR-146a. Furthermore, the levels of miR-146 in circulating plasma and exosomes were

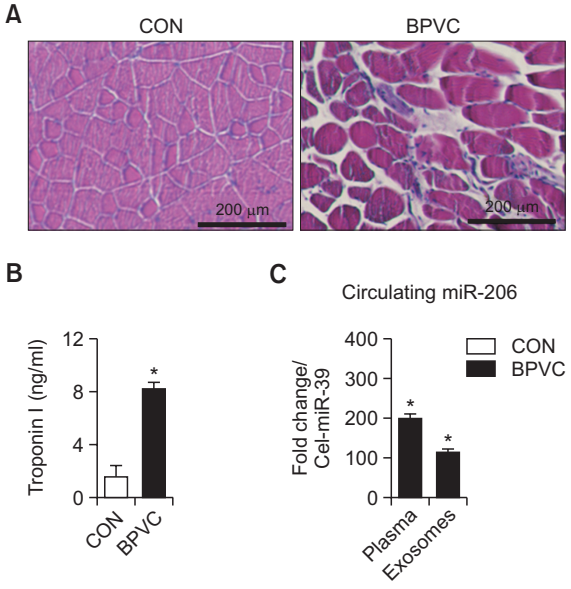

Fig. 5. Increased amounts of miRNA-206 in circulating exosomes in BPVC-induced muscle injury. (A) Representative images of hematoxylin and eosin (H&E) staining of formalin-fixed muscle sections. Scale bars, 200 μm. (B) Plasma levels of skeletal troponin I (n=5/group). (C) The amounts of miR-206 in circulating plasma and exosomes were quantified in CON- or BPVC-exposed mice (n=5/group). The data represent mean ± SD, **p*<0.05.

not elevated in APAP-induced liver injury (data not shown). Thus, these results support the utility of using exosomal miR-146a as a potential biomarker of kidney injury.

Elevation of circulating muscle-specific miR-206 in BPVC-induced muscle injury

To further support our hypothesis of using miRNAs as potential biomarkers for tissue-specific injury, we also determined the levels of circulating plasma and exosomal miR-206 in a mouse model of bupivacaine hydrochloride (BPVC)-mediated muscle injury. Here, using BPVC-induced muscle necrosis, where the sequences of fiber breakdown are similar to that of progressive muscular dystrophy (Nonaka *et al*., 1983). Histological analyses confirmed muscle injury after BPVC exposure (Fig. 5A). To assess the changes of muscle-specific miR-206 in the plasma and exosomes, we evaluated the expression of skeletal troponin I, known as a muscle injury marker protein, in the plasma from mice under BPVC-induced muscle injury. We observed the amounts of plasma troponin I were significantly elevated by BPVC exposure (Fig. 5B). Furthermore, the levels of plasma and exosomal miR-206 were markedly elevated in mice under BPVC-induced muscle injury (Fig. 5C). These results, observed for the first time, also validate the utility of using exosomal miR-206 as a potential biomarker of muscle injury.

DISCUSSION

MicroRNAs are expressed within cells in a tissue-specific manner and have been recently reported to be remarkably stable in plasma. Different miRNAs have emerged as fine regulators of gene function and their presence in various body fluids identifies them as attractive potential biomarkers of disease states (Etheridge *et al*., 2011). Extracellular miRNAs are associated with Ago complexes or packaged inside exosomes in cell lines or in healthy human plasma (Hu *et al*., 2012; Ohno *et al*., 2013). Although the identities, expression and functional roles of miRNAs have been extensively studied in the past, the content and role of a specific miRNA in circulating exosomes as potential biomarkers for tissue-specific injury have not been extensively evaluated. In fact, little is known about the fate of extracellular miRNAs after exposure to an organ-specific toxicant and their usage as potential biomarkers for tissue injury. Therefore, this study was aimed to examine the alterations of specific miRNAs in both circulating plasma and exosomes in the mouse models of drug-induced liver, kidney, or muscle injury. For the first time, our results showed that the levels of respective miRNAs in circulating plasma and exosomes were elevated in a few experimental models of drug-induced tissue injury. Our results also demonstrated that specific miRNAs may be able to detect hepatotoxicity, nephrotoxicity and myotoxicity in an organ-specific manner, since the elevated levels of plasma and exosomal kidney-specific miR-146 were not observed in APAP-induced liver injury, suggesting a tissue-specificity of a target miRNA.

For instance, we demonstrated that miR-122, miR-192, and miR-155 expression in circulating exosomes were elevated in a mouse model of APAP-induced liver injury. However, the potential usefulness and mechanisms underlying changes in liver expression of specific miRNA species, such as miR-122 and miR-192, which are enriched in liver tissue, remain to be elucidated. miR-155 is present in immune cells and hepatocytes (Faraoni *et al*., 2009). A recent study demonstrated increased miR-122 in the plasma of APAP-overdosed humans (Starkey Lewis *et al*., 2011). The authors reported time-dependent increases in plasma miR-122, miR-155, and miR-125b following APAP treatment (Wang *et al*., 2009). In addition, miR-122 and miR-155 are associated with exosome-rich fractions in a mouse model of alcohol- and inflammation-induced liver damage (Kaplowitz, 2005). N-acetylcysteine (NAC) was used to prevent APAP-induced hepatotoxicity (Chun *et al*., 2009). Ward and colleagues reported that NAC was effective at decreasing the elevated levels of circulating miRNAs in murine models of ischemic hepatitis and may be useful in other hepatotoxic conditions (Ward *et al*., 2014). Importantly, the levels of liver specific miRNAs were restored by NAC treatment used as an APAP antidote. These findings suggest that miRNAs become sensitive diagnostic biomarkers for liver injury in pre-clinic and clinic contexts.

The kidney-specific miR-146a was first identified in the immune system, where it regulates the mammalian response to microbial infection (Taganov *et al*., 2006). Additionally, Huang *et al*. (2014) reported that miRNA-155 and miR-146a were increased in the kidney tissues from patients with diabetic nephropathy and contribute to inflammation-mediated glomerular endothelial injury. Quercetin is one of the most abundant flavonoids in the human diet and exerts many beneficial effects on human health via its antioxidant properties and other effects (Boots *et al*., 2008). Lopez-Novoa and coworkers reported that quercetin markedly prevented cadmium- or cisplatin-induced nephrotoxicity, through its strong antioxidant and anti-inflammatory properties (Morales *et al*., 2006a, 2006b; Sanchez-Gonzalez *et al*., 2011). In our results, the amounts

of kidney-specific miRNA miR-146a in circulating exosomes were increased in a mouse model of CIS-induced kidney injury and its levels were returned to basal levels by quercetin (QR) treatment. Therefore, miR-146a is likely to represent a potential biomarker for drug-induced kidney injury, although further studies are needed to investigate function of miRNA, including miR-146a, in drug-induced kidney injury.

The skeletal muscle-specific miR-206 is required for efficient regeneration of neuromuscular synapses after acute nerve injury, and the absence of miR-206 accelerates disease progression of amyotrophic lateral sclerosis (ALS) in mice (Williams *et al*., 2009). Muscle-specific miRNA, miR-206 and other miRNAs (e.g., miR-1 and miR-133) induce expression of myogenic marker proteins after skeletal muscle injury and may play a crucial role in the regulation of muscle development and homeostasis (Nakasa *et al*., 2010). Liu *et al*. (2012) reported an essential role of miR-206 in satellite cell differentiation during skeletal muscle regeneration and indicated that miR-206 slows progression of Duchenne muscular dystrophy. To the best of our knowledge, our results represent the first report that identify circulating exosomal miR-206 as a potential biomarker of muscle injury in mice treated with BPVC.

Taken together, our current results demonstrated that specific miRNAs in circulating plasma and exosomes can be used as potential biomarkers for monitoring drug-induced liver, kidney, or muscle injury. However, the mechanism by which organ-specific miRNAs in circulating exosomes are elevated remains to be investigated. Our results with murine models of drug-induced tissue injury need to be further verified in appropriate human disease specimens.

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