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Circulating levels of miR-122 increase post-mortem, particularly following lethal dosing with pentobarbital sodium: implications for pre-clinical liver injury studies

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microRNA-122 (miR-122) is increasingly being measured in pre-clinical and clinical settings due to greater sensitivity and hepatic specificity compared to the gold standard liver injury biomarker alanine aminotransferase (ALT). In pre-clinical studies, various culling methods can be employed prior to collection of blood samples, including lethal injection with pentobarbital sodium (Pentoject). However, little is known about whether such an approach could alter the circulating levels of miR-122 and compromise the interpretation of data. We therefore exposed C57BL/6J mice to saline or the model hepatotoxin paracetamol and collected blood samples pre-cull (*via* tail bleed) and post-cull (*via* cardiac puncture following exposure to a rising concentration of CO_2 or intraperitoneal injection of Pentoject). Compared to pre-cull levels there was a significant increase in serum miR-122 level in mice culled with CO_2 and, to a much greater extent, in mice culled with Pentoject. As a result, whilst the serum level of miR-122 increased in Pentoject-culled animals exposed to paracetamol, the higher level in saline-treated mice rendered this difference statistically non-significant, in contrast to findings in animals culled with CO_2. ALT levels were unaffected by sacrifice method. Consistent with the *in vivo* findings, exposure of primary mouse hepatocytes to Pentoject provoked a rapid and concentration-dependent release of miR-122 into the culture media. Thus, for optimal design and interpretation of data from pre-clinical liver injury studies in which miR-122 is to be used as a biomarker, we recommend that blood samples are collected pre-cull whenever possible, and that lethal injection with Pentoject is avoided.

Introduction

Drug-induced liver injury (DILI) is an important human health concern.[1] Indeed, paracetamol (acetaminophen; APAP) overdose is the most frequent cause of DILI in the Western world, resulting in approximately 38 000 emergency hospital admissions per annum in England alone.[2] Moreover, DILI continues to impede the development of novel medications by the pharmaceutical industry. As a result, there is an increasing need for new, sensitive and organ-specific biomarkers to assess the safety of novel and/or existing drugs.[3] One such biomarker, microRNA-122 (miR-122), represents 75% of the total hepatic miRNA content and exhibits exclusive hepatic expression.[4] In pre-clinical and clinical settings, we and others have shown that miR-122 has higher sensitivity, with respect to dose and time, than alanine aminotransferase (ALT) and other clinical chemistry parameters as a biomarker of APAP-induced liver injury.[4–6] Due to its high clinical incidence, APAP-induced liver injury has been extensively studied in pre-clinical contexts.[7] Indeed, liver injury can be induced in rodents with a single dose of APAP, and several clinical phenotypes are well preserved in this animal model.[7,8] miR-122 is now commonly measured in these instances, alongside ALT, to detect liver injury at the earliest time point.[3,9] In such studies blood samples are commonly taken *via* tail bleed (pre-cull) or cardiac puncture (post-cull). In the latter case, one popular method of culling is *via* lethal injection with the anaesthetic pentobarbital sodium (Pentoject). In the field of veterinary anaesthesia it is known that extravascular administration of pentobarbital sodium can cause substantial tissue irritation.[10] However, little research has been carried out to investigate if there is an effect of this or other commonly-used culling methods on circulating levels of sensitive liver injury markers such as miR-122. Notably, Yu *et al.* and Cao *et al.* provided evidence for hepatotoxicity in rats following repeated high doses of Pentoject,[11,12] but there has yet to be any research into

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whether or not an acute effect could occur after a single lethal dose. Therefore, the aim of this investigation was to determine if lethal injection with Pentoject could affect serum miR-122 levels. Such knowledge is vital for the correct interpretation of existing biomarker data, and for the optimal design of future pre-clinical studies.

Materials & methods

Animal studies

All animal experiments were conducted according to the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. Male C57BL/6J mice (Charles River, UK), aged 7–9 weeks, were divided into three groups according to the treatment they received: saline (vehicle control), 35 mg kg^{-1} APAP (sub-toxic dose) or 300 mg kg^{-1} APAP (toxic dose). The choice of sub-toxic and toxic doses was based on historical ALT data. For all experiments, food was withdrawn 16 h prior to APAP administration, which was *via* intraperitoneal (IP) injection. After 24 h, tail bleeds were performed on all animals, and then half of the mice from each group were culled *via* exposure to a rising concentration of CO_2, with the other half culled *via* IP injection of 1000 mg kg^{-1} Pentoject. In both cases, blood was then immediately removed by cardiac puncture. Blood samples were left to clot at room temperature before separating the serum *via* centrifugation. Serum was stored at −80 °C until analysis, which excluded any haemolysed samples. Liver tissue samples were fixed in 4% paraformaldehyde before transferring to 70% ethanol for storage.

Murine primary hepatocyte experiments

Primary hepatocytes were isolated from C57BL/6J mice using a method previously described by Li *et al.*, 2010, with some minor modifications.[13] In brief, livers were perfused with wash buffer containing ethylene diamine tetraacetic acid followed by a digestion buffer containing $CaCl_2$ and type IV collagenase (Sigma-Aldrich, Missouri, USA). Hepatocytes were suspended in William's E medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 nM dexamethasone, 10 μg mL^{-1} insulin, 5.5 μg mL^{-1} transferrin, 6.7 ng mL^{-1} sodium selenite, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. The cells were seeded at 1×10^5 cells per cm^2 and incubated at 37 °C in a 5% CO_2 atmosphere. After 3 h the media and non-attached cells were removed and replaced with media lacking FBS. After a further 16 h the cells were exposed to the indicated concentrations of Pentoject for 5 min. Cellular ATP levels were determined using the CellTiter-Glo luminescent assay (Promega, Wisconsin, USA) according to the manufacturer's instructions. Cellular lactate dehydrogenase (LDH) levels were determined using the Cytotoxicity Detection Kit (Sigma-Aldrich) according to the manufacturer's instructions. The LDH content of the culture media could not be determined due to reaction of the assay reagent with high concentrations of Pentoject.

Quantification of miR-122 levels

miRNA was extracted and purified from 20 μL serum or 100 μL cell media using a commercially available miRNeasy kit followed by an RNeasy MinElute Cleanup Kit (Qiagen, Venlo, Netherlands), in accordance with the manufacturer's instructions. miR-122 level was measured using Taqman-based quantitative polymerase chain reaction (qPCR) as previously described.[3] Each miR-122 measurement involved duplicate qPCR repeats of the reverse transcription cDNA product as described by Sharkey *et al.*[14] For serum analysis, miR-122 Ct values were normalised to the level of endogenous let-7d. For culture media analysis, miR-122 copy numbers were calculated through the use of a standard curve generated with a synthetic miR-122 oligonucleotide. For the spiking experiment, a pool of control serum samples was directly supplemented with 25 mg mL^{-1} (estimated maximum blood concentration *in vivo*) or a 10-fold lower concentration of Pentoject prior to miRNA extraction, purification and quantification. These samples were tested in triplicate and an overall average recorded.

Quantification of ALT levels

Serum ALT levels were determined using a Thermo Infinity ALT Liquid Stable Reagent-based kinetic assay (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions and as previously described.[3]

Hepatic histological assessment

To enable histopathological assessment, fixed liver tissues were paraffin-embedded and 4 μm sections were stained with haematoxylin and eosin (H&E).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). A Shapiro Wilk test was used to assess normality of the data, followed by Mann–Whitney *U* tests or Unpaired *t* tests, as appropriate, to assess statistical significance, defined as $P < 0.05$.

Results

Lethal injection with Pentoject raises basal serum miR-122, but not ALT, levels

As expected, serum ALT levels were significantly increased in response to the toxic, but not sub-toxic, dose of APAP in animals culled by either rising CO_2 or Pentoject, when blood was sampled *via* cardiac puncture (Fig. 1A). There was no significant difference between the serum ALT levels in vehicle-treated mice culled by the two methods (Fig. 1A). There was a significant increase in serum miR-122 levels in response to the toxic dose of APAP in animals culled by rising CO_2 (Fig. 1B). However, there was also a significant increase in the level of serum miR-122 in vehicle-treated animals culled by Pentoject, compared with those culled by rising CO_2 (Fig. 1B). As a result, whilst the serum level of miR-122 increased in Pentoject-culled

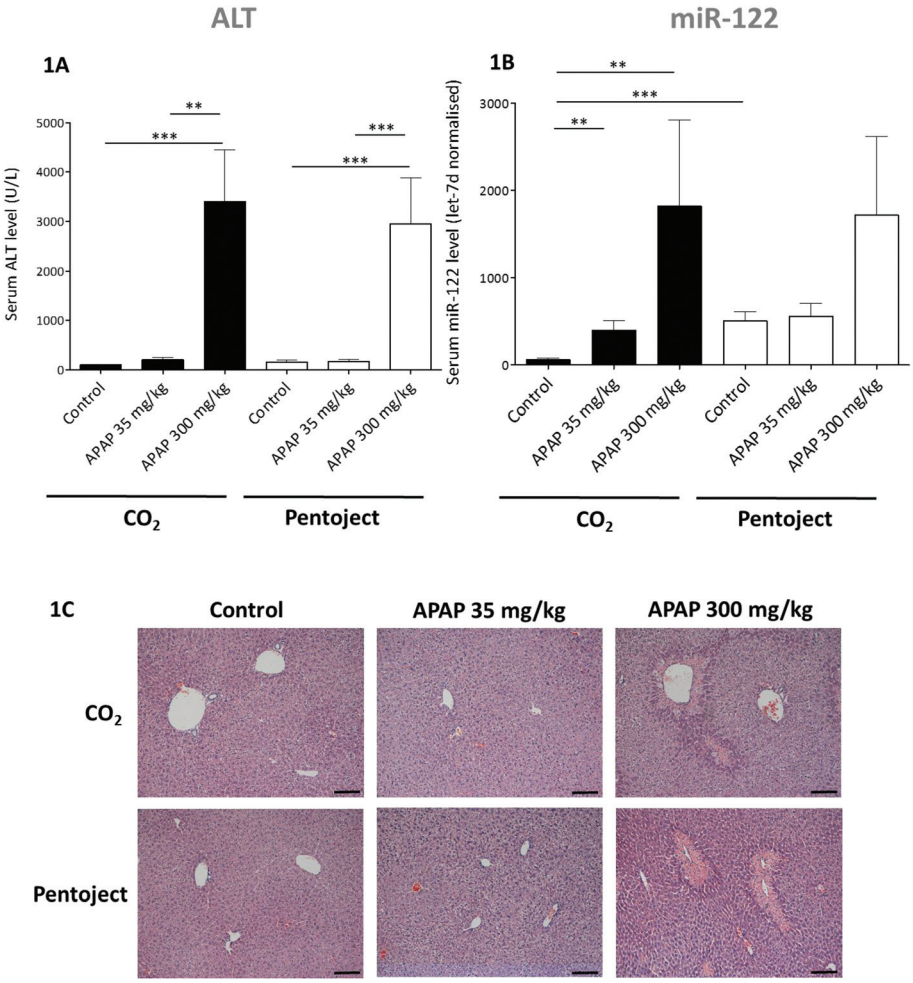

Fig. 1 Lethal injection with Pentoject raises basal serum miR-122, but not ALT, levels. Mice were exposed to vehicle control (saline), a sub-toxic dose (35 mg kg^{-1}) of APAP or a toxic dose (300 mg kg^{-1}) of APAP for 24 h. Blood samples were removed, *via* cardiac puncture, from mice following exposure to rising CO_2 or IP injection with a lethal dose of Pentoject. Serum levels of (A) ALT and (B) miR-122 were quantified, with the latter normalised to endogenous reference let-7d. Data is presented as mean ± SEM, with 9–12 animals per group. Statistical significance was determined by Mann–Whitney *U* test (***P* < 0.01, ****P* < 0.001). (C) H&E staining of representative livers from each group, showing marked centrilobular necrosis with the toxic dose of APAP. Scale bars indicate 500 μm.

animals exposed to the toxic dose of APAP, the higher level in vehicle-treated mice rendered this difference statistically non-significant (Fig. 1B). Therefore, Pentoject raises basal serum miR-122 level and impacts on the statistical interpretation of responses to APAP. Regardless of culling method, we observed multifocal to coalescing coagulative centrilobular necrosis in the livers of mice exposed to the toxic dose of APAP (Fig. 1C).

Serum miR-122 levels increase post-mortem, particularly following lethal dosing with Pentoject

To ensure that the higher basal serum level of miR-122 in animals culled with Pentoject was not an experimental artefact, we quantified the biomarker in the serum of the same animals sampled *via* tail bleed (*i.e.* pre-cull). Regardless of the culling method, serum miR-122 was significantly higher in samples obtained *via* cardiac puncture (*i.e.* post-cull) compared with those obtained *via* tail bleed (Fig. 2). Moreover, there was no difference in the levels of miR-122 in tail bleed samples of vehicle-treated mice that went on to be culled *via* rising CO_2 or Pentoject (Fig. 2), indicating that serum miR-122 levels rise post-mortem. However, the rise in serum miR-122 in animals culled with Pentoject was greater than that observed in animals culled with rising CO_2 (Fig. 2B), accounting for the statistical discrepancy in the miR-122 response to the toxic dose of APAP in blood samples obtained *via* cardiac puncture (Fig. 1B). Indeed, our analysis of tail bleed samples revealed a significant increase in serum miR-122 levels in response to the toxic dose of APAP in the animals that went on to be culled with Pentoject (Fig. 2B), indicating that the loss of statistical significance in samples collected post-cull was directly related to Pentoject.

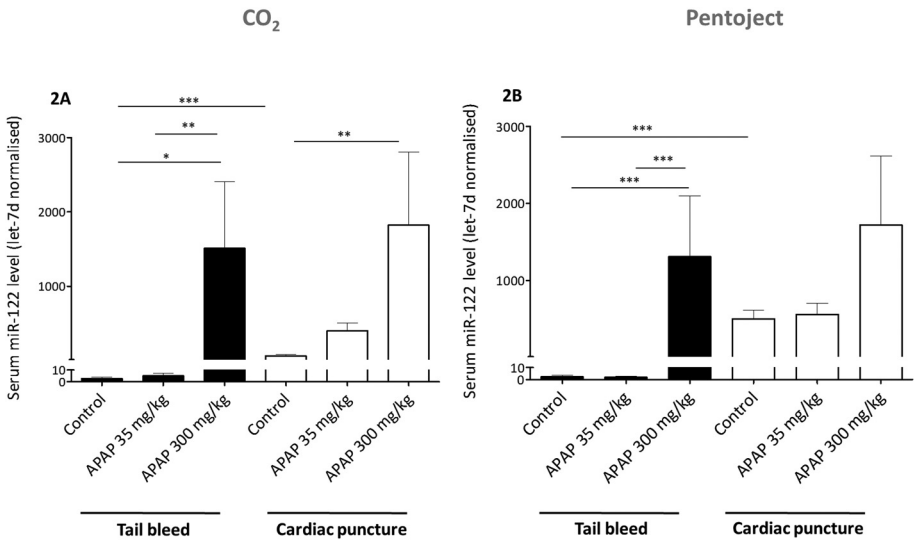

Fig. 2 Serum miR-122 levels increase post-mortem, particularly following lethal dosing with Pentoject. Mice were exposed to vehicle control (saline), a sub-toxic dose (35 mg kg^{-1}) of APAP or a toxic dose (300 mg kg^{-1}) of APAP for 24 h. Tail bleeds were performed prior to culling by (A) exposure to rising CO_2 or (B) injection with Pentoject. Serum levels of miR-122 were quantified and normalised to endogenous reference let-7d. Data is presented as mean ± SEM, with 9–12 animals per group. Statistical significance was determined by Mann–Whitney *U* test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Pentoject provokes the release of miR-122 from primary hepatocytes

We next isolated primary hepatocytes from mice and exposed them to Pentoject to further assess its ability to provoke the release of miR-122 from liver cells. Despite an extensive literature review, we were unable to find a maximum blood concentration of sodium pentobarbital following bolus IP injection of Pentoject. Therefore, we assumed that the 30 mg dose of Pentoject would be distributed within a total blood volume of 1.2 mL, giving a concentration of 25 mg mL^{-1}. As the local concentration was likely to be higher following IP injection, we exposed the hepatocytes to up to 100 mg mL^{-1} Pentoject for 5 min. At and above 10 mg mL^{-1} Pentoject provoked a significant and concentration-dependent increase in the number of miR-122 molecules in the culture media, concomitant with decreases in cellular ATP and LDH content, indicative of the loss of hepatocyte integrity (Fig. 3A). These data further confirm that Pentoject can provoke a rapid release of miR-122 from mouse liver cells.

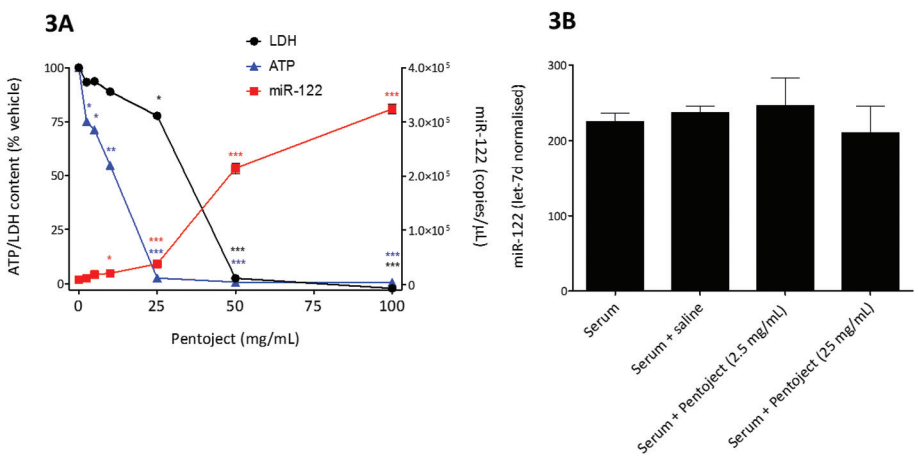

Fig. 3 Pentoject provokes the release of miR-122 from primary hepatocytes. (A) Levels of miR-122 in the media and cellular contents of ATP and LDH in primary mouse hepatocytes exposed to the indicated concentrations of Pentoject for 5 min. Data is presented as mean ± SEM of triplicate wells for a representative experiment. Statistical significance notifies differences between vehicle control (0 mg mL^{-1}) and other conditions, according to unpaired *t* tests (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). (B) miR-122 levels in serum spiked with Pentoject. miR-122 levels were quantified in pooled control serum directly supplemented with saline or the indicated concentrations of Pentoject. Data is presented as mean ± SEM of triplicate measurements. None of the groups were significantly different, according to Mann–Whitney *U* tests.

Pentoject does not cause assay-related perturbations

Finally, to confirm that the higher levels of miR-122 in samples obtained from Pentoject-culled mice were not assay-related, we performed extraction, purification, reverse transcription and qPCR analysis using a pool of control serum samples that was directly supplemented with saline or Pentoject. There was no change in the level of miR-122 in samples spiked with Pentoject (Fig. 3B), further indicating that Pentoject directly affects liver cells and causes an increase in circulating levels of miR-122.

Discussion

Given that DILI continues to be a clinical burden and barrier to the development of novel drugs, there is a clear need to qualify new biomarkers that can better predict human health hazard on the basis of pre-clinical studies and early phase clinical trials. Previously, we and others have shown that miR-122 is a more sensitive marker of DILI than ALT and other clinical chemistry parameters.[4–6] Moreover, recently, a panel of novel DILI biomarkers, including miR-122, received regulatory letter of support status from the US Food and Drug Administration (July 2016) and the European Medicines Agency (September 2016) for their further qualification as DILI biomarkers within a defined context of use. However, much remains to be learnt about miR-122 as a liver injury biomarker in different pre-clinical and clinical settings and as such, regulatory feedback has provided clear support for the incorporation of biomarkers such as miR-122 into exploratory DILI studies to further define whether such markers are fit-for-purpose.

Our current findings add significantly to the understanding of the utility of miR-122 as a putative DILI biomarker *in vivo*. We have demonstrated that, compared to pre-cull levels, there is a significant increase in serum miR-122 level in mice culled with CO_2 and, to a much greater extent, in mice culled with Pentoject. In the latter case, the increase in circulating miR-122 in control mice influences the statistical differentiation of the degree of liver injury following exposure to the model hepatotoxin APAP. The rise in circulating miR-122 after culling with CO_2 likely reflects early perturbations of the liver post-mortem whilst the rapid and direct effect of Pentoject provokes additional increases in the level of miR-122 in post-cull serum samples. Indeed, although our histopathological analysis did not reveal any overt perturbations that distinguished the livers of Pentoject and CO_2 culled animals, the *in vitro* experiments demonstrated that Pentoject can cause a rapid release of miR-122 from hepatocytes at concentrations that are relevant to those likely attained *in vivo*. Holman *et al.* recently provided evidence for the release of miR-122 within extracellular vesicles (such as exosomes) prior to overt cell death.[15] In contrast, ALT is known to be passively released into the serum following liver cell necrosis.[16] These observations may account for the apparent difference in sensitivity of the two markers to Pentoject-induced hepatic perturbation. Further work will be needed to clarify the precise mechanism underlying our observations. In addition, it will be important to determine whether miR-122 levels change in response to lethal doses of other anaesthetics that are used in pre-clinical studies, and whether the observed effect is conserved in other mouse strains and species.[17] Finally, it will be valuable to investigate whether the response of miR-122 is mirrored by that of other liver-related microRNAs, such as miR-192, as well as those associated with discrete organs such as the kidney.

Conclusion

The findings of this study highlight the need to carefully consider the method of culling used in pre-clinical studies that are designed to measure liver injury *via* the quantification of serum miR-122 levels. At least until further work has clarified the underlying pathological mechanism, we recommend that when miR-122 is to be used as a biomarker blood samples should ideally be collected pre-cull (*e.g.* by tail bleed). When this is not feasible, IP injection of Pentoject should be avoided as a culling method.

Conflict of interest

There are no conflicts of interest to declare.

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