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Letter

Protection from acetaminophen-induced hepatotoxicity by post-administration of 1*O*, 20*O*-diacetyl kamebakaurin in mice

Hiroki Yoshioka[1], Hiroyuki Ohnishi[2], Masae Yoshikawa[1], Ming-Yu Gui[3], Yong-Ri Jin[3], Xu-Wen Li[3], Yoshiyuki Adachi[4], Naohito Ohno[4], Koichi Takeya[4], Yukio Hitotsuyanagi[4], Nobuhiko Miura[5] and Yutaka Aoyagi[1]

[1]College of Pharmacy, Kinjo Gakuin University, 2-1723 Omori, Moriyamaku, Nagoya, Aichi 463-8521, Japan
[2]Department of Health Science, School of Allied Health Science and Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
[3]Department of Chemistry, JiLin University, No.2 Xinmin street, Changchun, JiLin 130021, People's Republic of China
[4]School of Pharmacy, Tokyo University of Pharmacy & Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[5]Division of Health Effects Research, Japan National Institute of Occupational Safety and Health, 6-21-1, Nagao, Tamaku, Kawasaki, Kanagawa 214-8585, Japan

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ABSTRACT — Our previous study demonstrated that pre-administration of 1*O*, 20*O*-diacetyl kamebakaurin (Ac_2KA) protected against acetaminophen (APAP)-induced hepatotoxicity. In the current study, we aimed to investigate whether post-administration of Ac_2KA also protects against APAP-induced hepatotoxicity. Eight-week-old male C57BL/6J mice were fasted and then intraperitoneally injected with 450 mg/kg APAP or saline. At 60-min after the APAP injection, Ac_2KA (50 mg/kg) or an ethanol/olive oil emulsion was orally administered. At 16-hr after the injection, the mice were killed, and blood samples were collected for plasma analysis. As a positive control, we used N-acetylcysteine (200 mg/kg, i.p.). Post-treatment with Ac_2KA significantly attenuated APAP-induced plasma alanine aminotransferase and aspartate aminotransferase levels. Ac_2KA administration also decreased the APAP-induced hepatic malondialdehyde concentration. Moreover, histological evaluation supported these observations. Our results show that Ac_2KA exerts protective effects against APAP-induced hepatotoxicity when administered as both pre-treatment and post-treatment.

Key words: Acetaminophen, 1*O*, 20*O*-diacetyl kamebakaurin, Oxidative stress, Hepatic injury

INTRODUCTION

Acetaminophen (APAP) is recognized as a popular antipyretic and analgesic drug. Although APAP is thought to be safe at low doses, an overdose is known to induce severe hepatic injury in humans and laboratory animals (McGill *et al*., 2012). APAP overdose has become the most frequent cause of acute hepatic injury in many countries (Schiodt *et al*., 1997). Several studies have established that the formation of the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which first depletes glutathione levels and subsequently causes protein binding, is a critical event in the toxicity (Black, 1984).

Based on this mechanism, *N*-acetylcysteine (NAC) was prescribed to treat APAP overdose patients in the 1970s. Even today, NAC is the best therapeutic option for these patients. The mechanism of NAC action is to promote

Correspondence: Hiroki Yoshioka (E-mail: h-yoshioka@kinjo-u.ac.jp)
Yutaka Aoyagi (E-mail: yutaka@kinjo-u.ac.jp)

hepatic glutathione synthesis (Mazer and Perrone, 2008), which supports the detoxification of NAPQI and reduces protein binding. Clinical studies have reported that NAC often induces undesirable side effects (Smilkstein *et al*., 1988). Therefore, it is essential to develop side-effect free therapeutics that offer maximum protection against APAP-induced hepatic injury.

Our previous investigation indicated that 1*O*, 20*O*-diacetyl kamebakaurin (Ac_2KA), which is synthesized from kamebakaurin, prevents APAP-induced hepatic injury by inhibiting lipid peroxidation and inflammatory responses (Yoshioka *et al*., 2018). Although Ac_2KA was found to exert a good protective effect, our previous study only evaluated the pre-treatment effects of Ac_2KA. It is also important to examine the protective effect of Ac_2KA post-administration against APAP-induced hepatotoxicity. If post-treatment with Ac_2KA is effective against APAP-induced hepatic injury, Ac_2KA might be suitable as an alternative to NAC.

Therefore, we investigated the protective mechanisms of Ac_2KA post-administration against APAP-induced hepatic injury.

MATERIALS AND METHODS

Animal treatment

Male 7-week-old C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Following arrival at our facility, the mice were maintained under standard conditions of controlled temperature (23 ± 2°C), humidity (55 ± 5%), and light (12-hr light/dark cycle) with free access to food and water. Experiments were conducted using 8-week-old mice. These experiments were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University.

Experimental protocol

The mice were randomly divided into 4 groups of 5-7 mice each. Prior to APAP injection, the mice were fasted for 16-hr, and mice in the APAP group, the APAP + Ac_2KA group, and the APAP + NAC group were intraperitoneally injected with 450 mg/kg APAP in an emulsion of polyethylene glycol and saline. The control group mice received a polyethylene glycol and saline emulsion. Sixty minutes after APAP administration, the APAP + Ac_2KA group received 50 mg/kg Ac_2KA orally in an olive oil/ethanol emulsion. As negative controls, both the control and APAP groups were injected with the olive oil/ethanol emulsion. As a positive control, the APAP + NAC group was intraperitoneally injected with 200 mg/kg NAC. After 16-hr of APAP administration, mice from each group were killed by pentobarbital overdose and bled for plasma isolation. The plasma was stored at -80°C until analysis. In addition, the livers were rapidly removed, and the tissues were fixed in a formalin solution for histological analysis. Other portions of the liver specimen were snap-frozen in liquid nitrogen and subsequently stored at -80°C.

Plasma biochemical analysis

Plasma alanine aminotransferase (ALT) and asparate aminotransferase (AST) activities were measured using the Transaminase CII Test Wako (Wako Pure Chemicals, Osaka) according to the manufacturer's instructions. Each plasma sample (1.5 μL) was mixed with the substrate (75 μL), incubated at 37°C for 15 min, and then quenched by adding the stopping solution (150 μL). The absorbance of blue pigment formed by the reaction was measured at 555 nm (Yoshioka and Onosaka, 2016).

Measurement of malondialdehyde levels in the liver

Total malondialdehyde (MDA) levels in the liver were examined via a colorimetric microplate assay (Oxford Biochemical Research, Oxford, MI) according to the manufacturer's protocol and previously described (Fukaya *et al*., 2018).

Histopathological findings

A portion of the left lobe from each liver was fixed in a formalin solution, dehydrated, and embedded in paraffin. Sections (4-μm) were cut from the paraffin-embedded blocks. The sections were dewaxed in xylene and rehydrated in a graded ethanol series. After rehydration, the sections were stained with Mayer's hematoxylin solution (Wako Pure Chemical). After rinsing in running tap water, the sections were stained with a 0.1% eosin solution (Wako Pure Chemical) containing acetic acid. Finally, the sections were dehydrated, cleared, and mounted with a cover glass. The histopathological features of each section were examined under a light microscope.

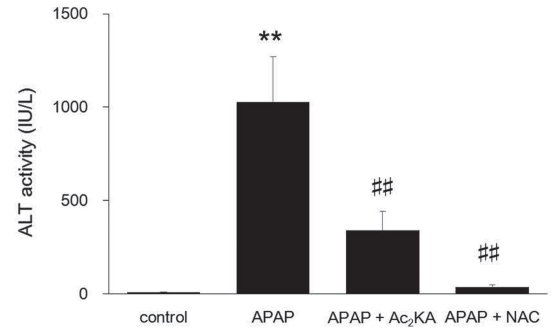
Statistical analysis

Statistical analyses of multiple comparisons were performed using a one-way analysis of variance with the Tukey's post-hoc test. All statistical analyses were performed using the SPSS 24.0 software (SPSS, Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

RESULTS

To begin our analysis, we determined the plasma

(A)



(B)

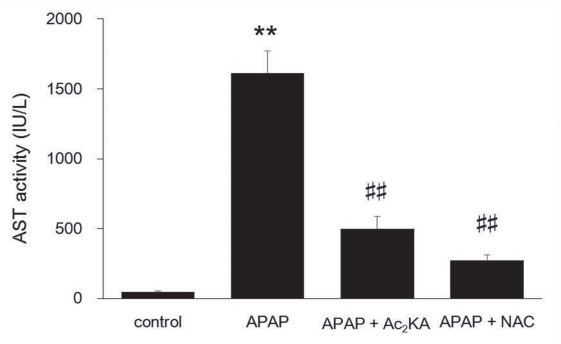


Fig. 1. Effect of Ac_2KA post-treatment on hepatic injury marker levels. Panels (A) and (B) indicate plasma ALT and AST activity levels, respectively. Data are presented as the mean ± S.D. (n = 5-7 per group). ** *p <0.01* versus control group, and ## *p <0.01* versus APAP group.

ALT and AST activity levels. Exposure to APAP led to a significant increase in ALT and AST activity at 16 hr (Fig. 1A and 1B). Post-treatment with Ac_2KA significantly decreased the APAP-induced ALT and AST activity. Compared to NAC, the level of repression of AST was comparable but the level of ALT repression was lower.

We measured liver MDA levels as a marker of lipid peroxidation (Fig. 2). APAP administration significantly increased hepatic MDA levels ($p < 0.01$), whereas post-treatment with Ac_2KA significantly attenuated the APAP-induced upregulation in MDA levels ($p < 0.05$). Use of NAC resulted in greater attenuation than Ac_2KA.

Finally, we evaluated liver histopathology using H&E

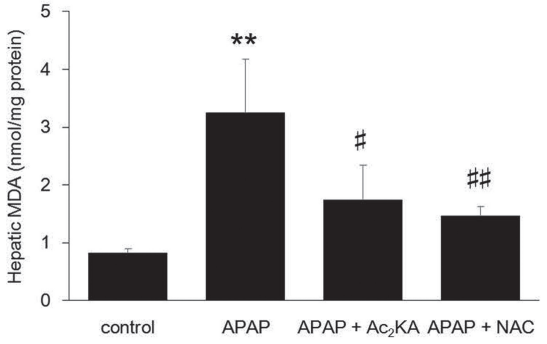


Fig. 2. Effect of Ac_2KA post-treatment on hepatic MDA levels. Data are presented as the mean ± S.D. (n = 5-7 per group). ** *p <0.01* versus control group, and # *p <0.05* and ## *p <0.01* versus APAP group.

staining (Fig. 3). The livers of APAP-treated mice showed severe necrosis (Fig. 3B). The livers of mice post-treated with Ac_2KA also showed necrosis; however, the necrosis level, as measured by the necrotic area, was lower than that in the APAP-treated group (Fig. 3C). In mice post-treated with NAC, no necrosis was observed (Fig. 3D).

DISCUSSION

This study showed that Ac_2KA post-administration inhibited APAP overdose-induced acute toxicity in the mouse liver. In this analysis, we used NAC as a positive control since it is used to treat APAP overdose patients. Compared to NAC, treatment with Ac_2KA was found to have a diminished protective effect.

Several studies have indicated that the main mechanism of NAC action is to increase hepatic glutathione levels (Terneus *et al*., 2008; Mazer and Perrone, 2008). Our preliminary study demonstrated that the antioxidant potential of Ac_2KA was not the result of glutathione upregulation (data not shown). These data suggest that Ac_2KA was not increasing glutathione. One possible mechanism to explain our results involves the c-Jun N-terminal kinase inhibitor. Recently, this inhibitor has been implicated in playing a role related to APAP-induced hepatotoxicity (Henderson *et al*., 2007; Schwabe *et al*., 2003). Since our previous analysis showed that APAP-induced c-Jun N-terminal kinase phosphorylation is attenuated by Ac_2KA (Yoshioka *et al*., 2018), this hypothesis is plausible. Saito *et al*. showed that NAC post-administration has two effects: enhancing hepatic glutathione levels and support-

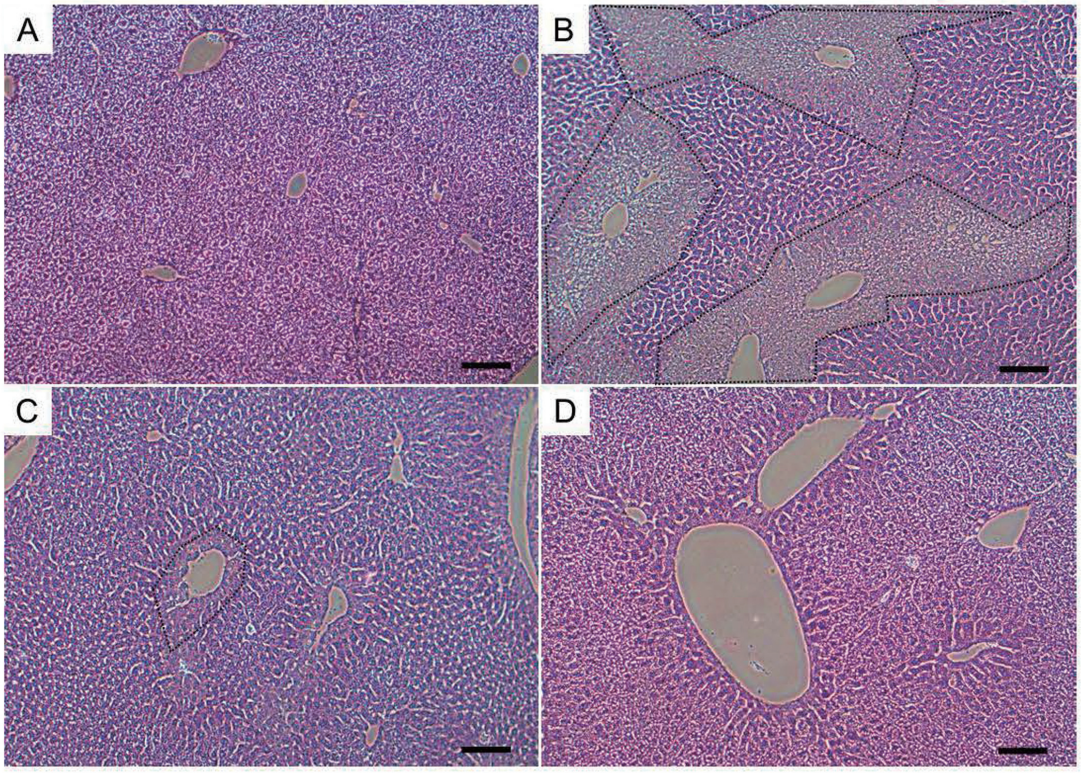


Fig. 3. Ac_2KA post-treatment protects against APAP-induced hepatotoxicity, as assessed by H&E staining. Panels (A) – (D) indicate the control, APAP, APAP + Ac_2KA, and APAP + NAC groups, respectively. Panel (B) shows severe necrosis and panel (C) shows mild necrosis. Bar, 100 μm.

ing mitochondrial energy metabolism (Saito *et al*., 2010). Because our current analysis does not focus on mitochondrial energy metabolism, additional studies are needed to fully understand the molecular protective mechanism of Ac_2KA.

In conclusion, we showed that both pre-treatment and post-treatment with Ac_2KA have protective effects against APAP-induced hepatic injury. The administration routes and dosages were different for NAC and Ac_2KA. NAC was administered by intraperitoneally injection at four-times the dose level of Ac_2KA, which was given orally. Therefore, a direct comparison of the individual agents was not possible, Ac_2KA might be considered an alternative medication to NAC. Further studies are needed to elucidate the precise protective mechanism of Ac_2KA. We believe our current findings contribute to the development of treatments against acute liver injury and disease.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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