



# Subcutaneous toxicity of melittin-dKLA in ICR mice

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## Abstract

**Background** Melittin, a major component of honeybee venom, is known to possess anti-allergic, anti-inflammatory, anti-arthritis, and anti-cancer. Despite its great promise as an agent for various diseases, the therapeutic applications of melittin have been severely limited by its nonspecific cytotoxicity and hemolytic activity.

**Objective** We synthesized a hybrid peptide (melittin-dKLA), composed of melittin and the pro-apoptotic peptide (dKLA), and evaluated its potential toxicity in ICR mice following a single or repeated subcutaneous dose administration.

**Results** Mice administered a single subcutaneous dose of melittin-dKLA showed no mortality or abnormal clinical signs in all animals. Similarly, mice administered a repeated subcutaneous dose of melittin-dKLA resulted in no deaths. Crust formation and induration were observed in the male and female mice in the 5 and 20 mg/kg, respectively. The hematological parameters and serum biochemical analysis did not show any changes by melittin-dKLA. In repeated subcutaneous dose study, the mice in the 20 mg/kg group showed a significant increase in the relative weight of the spleen compared to the control group. Although the difference was not statistically significant. Mice exhibited a common pattern of change.

**Conclusion** Taken together, our results suggest that the toxicity of melittin-dKLA is higher than 20 mg/kg both as a single and a repeated subcutaneous administration in ICR mice. Thus, it is suggested that it will be an important data in the development of new drug for melittin-dKLA.

**Keywords** Melittin-dKLA · Toxicity · Clinical sign · Hematology · Necropsy

## Introduction

Melittin is the main component (approximately 40–50% of dry weight) of honey bee (*A. mellifera*) venom and a cationic peptide with a sequence of 26 amino acids: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln [NH<sub>2</sub>-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH<sub>2</sub>]

(Aufschnaiter et al. 2020), with the chemical formula C<sub>131</sub>H<sub>228</sub>N<sub>38</sub>O<sub>32</sub> (MW 2847.5 Da) (Alonezi et al. 2017; Somwongin et al. 2018). Bee venom therapy has been traditionally used for the treatment of a variety of ailments, including arthritis, rheumatism, pain, tumors, and skin diseases (Carpena et al. 2020). In particular, melittin in bee venom has been reported to have exert biological effects, including anti-parasitic, anti-bacterial, anti-viral, and anti-inflammatory activities, in several studies (Jamasbi et al. 2016; Lee et al. 2016). In addition, melittin has been identified to be useful in antitumor therapy through in vitro studies on the number of cancer cells, including human ovarian cancer cells (SKOV3 and PA-1), human melanoma (A2058), prostate (PC-3, LNCaP, and DU-145), cervical cells (HeLa), and human leukemia cells (U937) (Gajski et al. 2013; Jo 2012; Park 2011; Therrien et al. 2016; Wang 2009). In our previous study, melittin was found to suppress tumor progression in a Lewis lung carcinoma mouse model (Lee et al. 2017). The current literature involving both in vitro and in vivo studies has suggested that melittin affects signal transduction and regulatory pathways

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leading to multiple cancer death mechanisms, including the inhibition of proliferation, the induction of apoptosis, the inhibition of angiogenesis, cell cycle arrest, and the inhibition of cancer motility, migration, metastasis, and invasion (Rady et al. 2017). One of the potential uses of melittin in anticancer therapy is through its induction of necrotic cell death in cancer cells via the activation of caspases and matrix metalloproteinases (Gajski et al. 2013). Although it is an attractive therapeutic agent for cancer, as well as various human diseases, melittin has been known to induce extensive hemolysis, a severe side effect that dampens any future development and clinical applications (Lyu et al. 2019).

Melittin is known to bind to negatively charged membrane surfaces and disturb the integrity of phospholipid bilayers via pore formation, accompanied by the leakage of atomic ions and molecules, and the enhancement of permeability, ultimately leading to cell lysis (Jamasbi et al. 2016; Rady et al. 2017). On the other hand, low doses of melittin have been reported to be able to prevent pore formation (van den Bogaart et al. 2008). Moreover, melittin has been reported to show a LD50 value of 2.5–5 mg/kg when administered intravenously in a mouse model (Zhou 2021). However, melittin has not been reported for toxicity when injected subcutaneously, which is less toxic than intravenous injection. Therefore, it is necessary to confirm the appropriate concentration of melittin through toxicity tests in the development of therapeutic agents for various diseases.

We previously synthesized a fusion peptide of the proapoptotic peptide dKLA (all-D enantiomer form) via a GGGGS linker conjugated to melittin for the development of anticancer drugs for breast cancer (Lee 2019). In the synthetic peptide, the cationic and amphipathic  $\alpha$ -helix peptide (KLAKLAK)<sub>2</sub> (KLA) as mitochondrial membrane-disrupting agent is a naturally occurring antibacterial peptide that binds to and disrupts the negatively charged bacterial membrane. A characteristic of this peptide is the fact that it does not cross the zwitterionic eukaryotic plasma membrane, and therefore does not exhibit cytotoxicity to eukaryotic cells (Foillard 2008; Javadpour 1996). Accordingly, KLA must be fused with other peptides to facilitate the ability of eukaryotic cells to disrupt membranes (Kim, 2011; Kwon, 2008; Law et al. 2006; Mai et al. 2001). KLA peptides internalized into the plasma membrane induce programmed cell death by disrupting the negatively charged mitochondrial membrane and inducing the release of cytochrome c (Ellerby 1999). However, a toxicity test for synthetic peptides has yet to be conducted.

In this study, we aimed to assess the toxicity and the approximate values of the lethal dose of melittin-dKLA following a single subcutaneous dose administration in ICR mice, as well as evaluate the potential toxicity of

melittin-dKLA subcutaneously administered in ICR mice every other day for 4 weeks.

## Materials and methods

### Animals

ICR wild-type mice (male and female, 6 weeks old) were purchased from Orient Bio Inc. (Seongnam, Korea). The animal procedures were approved by the Institutional Animal Care and Use Committee (19M041) in accordance with the “Animal Protection Act” (no. 16075, revised on March 25, 2019). Environmental controls were set at the following conditions: temperature range,  $23 \pm 3$  °C; relative humidity range,  $55 \pm 15\%$ ; ventilation, 10–20 air changes/h; luminous intensity, 150–300 lx; 12/12 h light/dark cycle.

### Experimental design

A single subcutaneous dose of melittin-dKLA was administered in ICR mice to assess its general toxicity ( $n = 5/\text{sex}/\text{dose group}$ ) at 0, 5, 10, and 20 mg/kg. Melittin-dKLA was dissolved in saline and administered subcutaneously once in ICR mice. After the injection of melittin-dKLA, several parameters were observed, including mortality, clinical signs, body weight changes, and necropsy, for 14 days. For the repeated subcutaneous administration of melittin-dKLA, we were carried out according to Organization for Economic Cooperation and Development (OECD) test guideline (OECD Guideline 407) with slight modifications (OECD 2008). The ICR mice ( $n = 5/\text{sex}/\text{dose group}$ ) were subcutaneously injected with 0, 1.25, 5, and 20 mg/kg of melittin-dKLA repeatedly (every other day) for 4 weeks. Similar to the procedure in the single dose group, several parameters were observed, including mortality, clinical signs, body weight changes, food consumption, hematology and serum biochemistry, necropsy findings, and relative organ weights, in mice administered repeated doses. This study was conducted in accordance “Guideline for Toxicological Test”, Ministry of Food and Drug Safety (notification no. 2017–71) (Aug. 30, 2017).

### Body weight

The body weight of the mice administered a single subcutaneous dose of melittin-dKLA was measured upon their acquisition, group assignment, prior to dosing (day 0), and on days 1, 3, 7, and day 14 (necropsy) using an electronic balance (ENTRIS2202-1S; Sartorius, Germany). In the mice-administered repeated subcutaneous doses, the body weight was recorded upon receipt, prior to dosing on day 1, once a week during the dosing period, on the day prior to

necropsy, and on the day of necropsy using an electronic balance (Sartorius). Body weight data on the day of necropsy were not included in the evaluations of body weight, since these data represent the fasted body weights of all animals.

### Food consumption

Food consumption was recorded once a week from the first day of dosing to the day after dosing using an electronic balance (Sartorius). During the study period, the mean daily food consumption was calculated using the food consumption for 1 day, which was recorded once a week.

### Clinical pathology

For blood sampling, all surviving animals were fasted for approximately 4 h prior to necropsy. Prior to the collection of blood samples, the animals were anesthetized with isoflurane (Lot No.: N0368D25), and blood samples were collected from the abdominal aorta. Blood samples for hematology and clinical chemistry were collected and placed in a vacutainer containing K2-EDTA tubes. The following parameters were analyzed using a blood cell analyzer (ADVIA 2120i; Siemens, Germany). Then, roughly mixed blood with 3.2% sodium citrate was centrifuged at 3,000 rpm for 10 min at 4 °C to obtain plasma. The following parameters were measured in seconds from the plasma using the nephelometric analysis method with a coagulation time analyzer (ACL 100; Instrumentation Laboratory, USA): total erythrocyte count (RBC), RBC distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean platelet volume (MPV), hematocrit (HCT), hemoglobin distribution width (HDW), hemoglobin (HGB), total leukocyte count (WBC), WBC differential count, neutrophil (NEU), lymphocyte (LYM), monocyte (MONO), eosinophil (EOS), basophils (BASO), large unstained cells (LUC), platelets (PLT), and reticulocytes (Retic).

For clinical chemistry, the following parameters were analyzed using a blood biochemical analyzer (Hitachi 7180; Hitachi, Japan): aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose (GLU), blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (T-CHO), creatine kinase (CK), total protein (TP), albumin (ALB), total bilirubin (T-BIL), triglycerides (TG), A/G ratio (A/G), and calcium (Ca).

### Tissue pathology

Regarding the weight of the organs of the sacrificed animals, the following organs were weighed individually: kidney, thymus, heart, liver, adrenal, spleen, epididymis, testis, prostate, seminal vesicle, ovary, uterus, and ling. Paired organs,

seminal vesicles, and prostates were weighed together. Organ-to-body weight (terminal body weight) ratios were calculated based on fasted body weights.

### Statistical analysis

The statistical parameters were as follows: body weight, hematological parameters, serum biochemical analysis, and absolute and relative organ weights. Parametric multiple comparison procedures were subjected to one-way analysis of variance (ANOVA) for significance, which was judged at a probability value of  $p < 0.05$ . If a test showed a significant difference, it was subjected to Levene's test for equality of variances. If homoscedasticity of the data was accepted, the Scheffe multiple range test was applied. In cases where a significant deviation of variance homogeneity was observed, Dunnett's T3 test was applied for post hoc analysis. Parametric or non-parametric multiple comparison procedures were used for comparison. All statistical analyses were performed using the widely used commercial statistical package SPSS software (IBM® SPSS® Ver. 24).

## Result

### Changes in body weight and mortality after single subcutaneous administration of melittin-dKLA in ICR mice

To determine whether the body weight of mice decreased after a single subcutaneous administration of melittin-dKLA, male and female ICR mice were administered doses of 0, 5, 10, and 20 mg/kg, and changes in their body weight were observed. During the observation period, a decrease in body weight was observed in males on day 1 and in females on day 1 and day 3 after dosing in the control group (0 mg/kg). These animals returned to normal on day 3 and 7. A decrease in the body weight was evident in females on day 14, while a decrease in body weight was evident in males on days 1 and 7, and in females on day 1, day 3, and day 7 after dosing in the low-dose group (5 mg/kg). The body weight of these animals returned to normal on days 3, 7, and 14, respectively. A decrease in body weight was observed in males on day 3 and 7, and in females on day 1 and 3 after dosing in the middle dose group (10 mg/kg). These animals returned to normal on day 7 and 14, respectively. A decrease in the body weight was evident in males and females on days 1 and 3 after dosing in the high-dose group (20 mg/kg). These animals returned to normal on day 3, 7, and 14, respectively. A decrease in body weight was observed in males on day 14 (Fig. 1A, B). Similar reductions in weight were also observed in the control group, and the weight of the mice treated with melittin-dKLA did not show

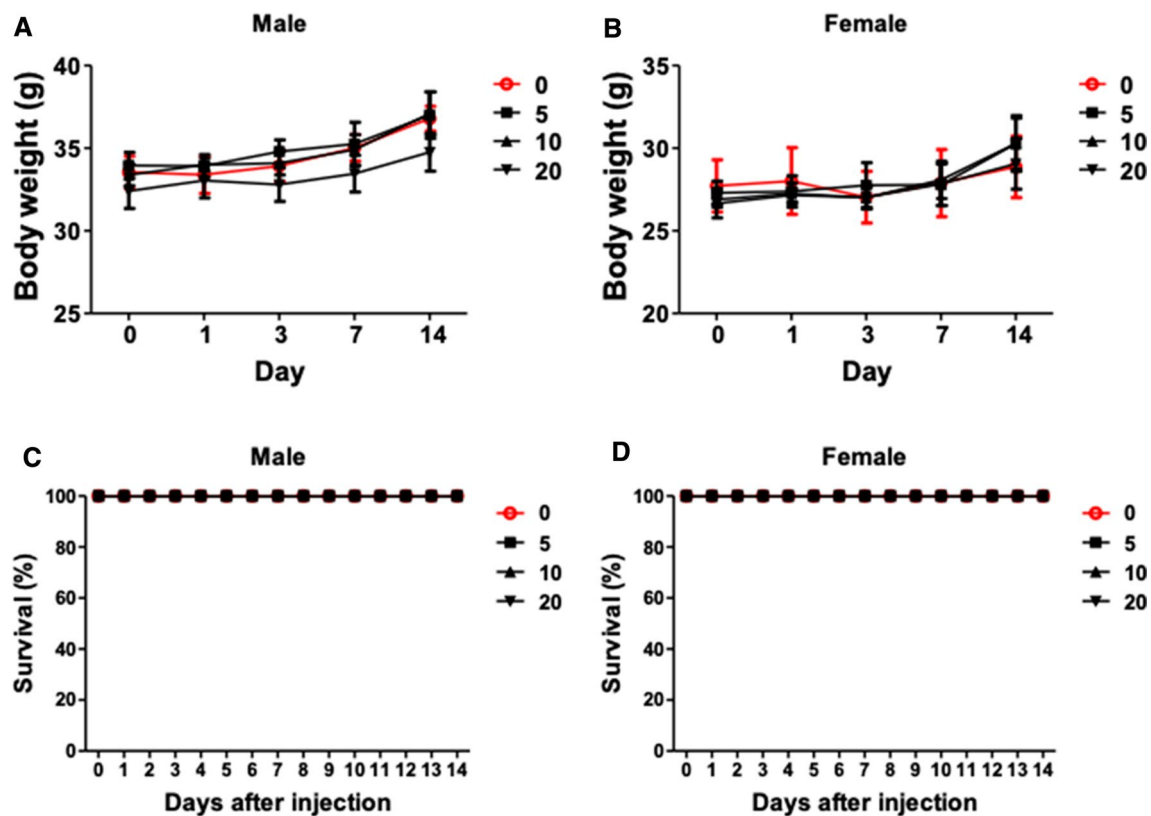


**Fig. 1** Changes in body weight and mortality in ICR mice after a single subcutaneous administration of melittin-dKLA. ICR mice (6 weeks old,  $n=5$ /sex/dose group) were subcutaneously administered melittin-dKLA with doses of 0, 5, 10, and 20 mg/kg. The body weights of male (A) and female (B) mice were measured prior to dos-

ing (day 0) and on day 1, 3, 7, and 14 using an electronic balance. The mortality of male (C) and female (D) mice was observed on day 0 and once a day thereafter for 14 days. The data shown are the mean  $\pm$  SD

a significant decrease compared to the control group. Thus, these results were considered to be temporary symptoms rather than an effect of the test substances.

To observe the mortality of ICR mice administered melittin-dKLA, male and female mice were administrated subcutaneously once with doses of 0, 5, 10, and 20 mg/kg of melittin-dKLA and observed daily. Mortality was not observed during the observation period in any of the animals (Fig. 1C, D).

To evaluate the abnormality of clinical signs observed after a single subcutaneous administration of melittin-dKLA, male and female ICR mice were first administered doses of 0, 5, 10, and 20 mg/kg and then observed for clinical signs 30 min after dosing, and at 1, 2, 3, and 4 h after dosing on day 0 and once a day thereafter for 14 days. However, no abnormalities were observed in all mice for observation period (Table S1A-D). After the end of the observation period, complete gross postmortem examinations were performed. As a result, no grossly visible findings were evident in any of the groups (Table S1E and F). Thus, these results suggest that melittin-dKLA does not affect the abnormality of clinical signs and necropsy findings in a single

subcutaneous administration in ICR mice and the values of a lethal dose of melittin-dKLA are above 20 mg/kg.

### Body weight change, food consumptions and mortality by repeated subcutaneous administration of Melittin-dKLA in ICR mice

To determine whether melittin-dKLA induces a decrease in body weight following repeated subcutaneous administration, ICR mice were administered doses of 0, 1.25, 5, and 20 mg/kg, and the body weights were observed. A statistically significant difference in the mean body weight was not observed for males or females in all treated groups compared to the control during the dosing period (Fig. 2A, B). Additionally, we evaluated the amount of food intake for the toxicity of melittin-dKLA in ICR mice. An increased food consumption was observed in males in the 20 mg/kg group and in females in the 20 mg/kg group compared to the control group (Fig. 2C, D). However, the mortality of ICR mice after melittin-dKLA administration did not show abnormal findings in any of the dose groups (Fig. 2E, F).

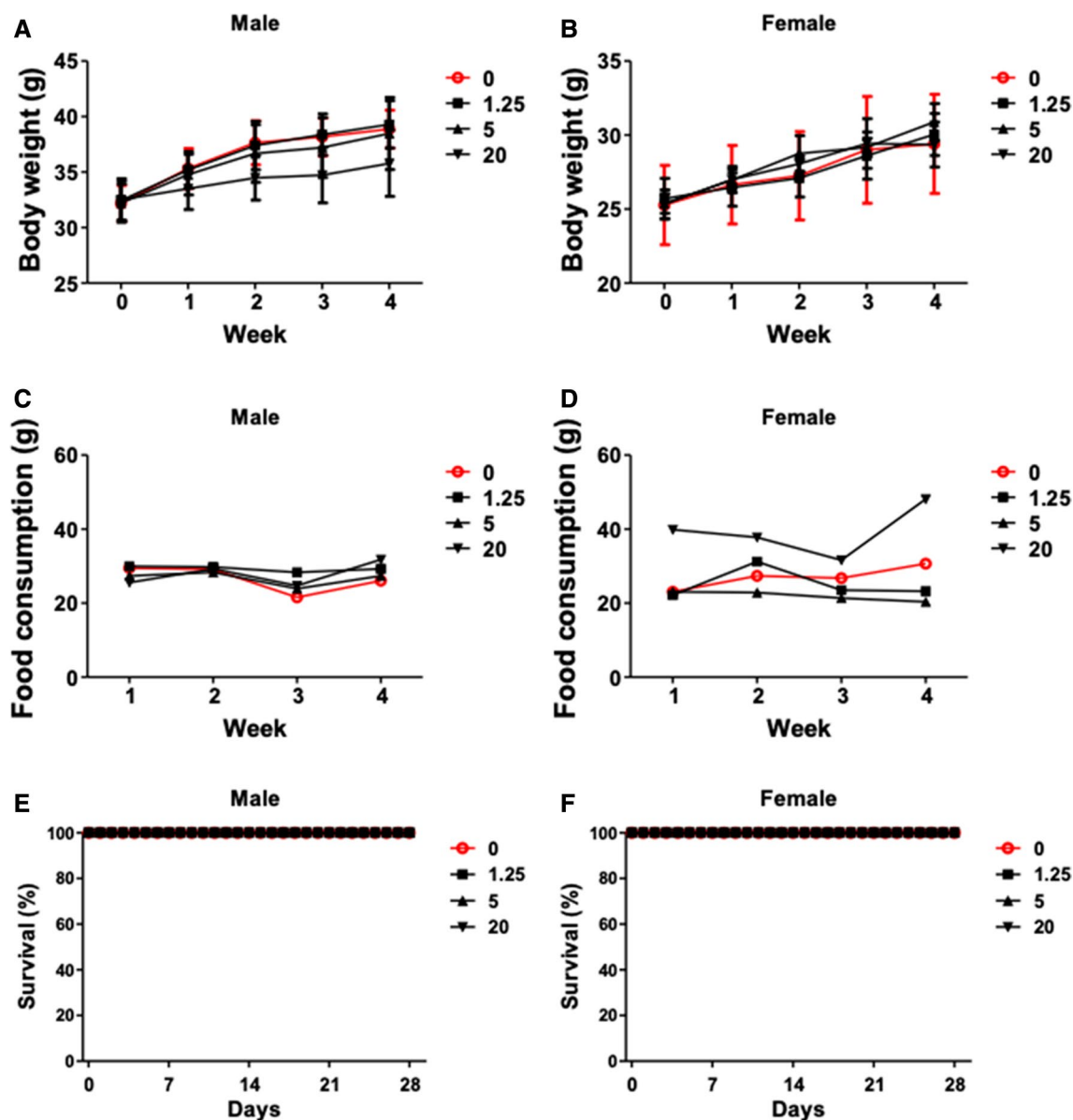


**Fig. 2** Changes in body weight, food consumption, and mortality in ICR mice after the repeated subcutaneous administration of melittin-dKLA. ICR mice (6 weeks old,  $n=5$ /sex/dose group) were subcutaneously administered melittin-dKLA with doses of 0, 1.25, 5, and 20 mg/kg. The body weight of male (A) and female (B) mice was measured prior to dosing on day 1, once a week during the dosing

period using the electronic balance. Food consumption by male (C) and female (D) mice was recorded once a week from the first day of dosing to the next day of dosing using an electronic balance. The mortality of male (E) and female (F) mice was observed weekly for 4 weeks. The data shown are the means  $\pm$  SD

To evaluate the abnormality of the clinical signs in ICR mice after the repeated subcutaneous administration of melittin-dKLA, male and female mice were administered doses of 0, 1.25, 5, and 20 mg/kg and clinical signs were observed once a day for 28 days. In the male mice in the 5 mg/kg group, crust formation was observed in one case on day 9 of administration. On day 17, induration was observed in one case for the first time. The two symptoms persisted until the end of the administration. In the 20 mg/kg group,

crust formation was observed in two cases on day 9. On day 17, three cases of crust formation and one case of induration were observed. Moreover, day 25, four cases of crust formation were observed. Similarly, in the 5 mg/kg group, any observed symptoms persisted until the end of administration (Table S2A). In the female mice in the 5 mg/kg group, two cases of crust formation were observed for the first time on day 9. Moreover, one case was observed on day 13, and two cases were observed on day 25. Additionally, induration was

observed in one patient on day 17. These symptoms were continuously observed until the end of administration. In the 20 mg/kg group, three cases of crust formation were observed for the first time on day 9, and all five cases were observed to have crust formation on day 11. In addition, induration was observed in two cases on day 17, and these symptoms were observed continuously until the end of the administration, as in other groups (Table S2B). In the necropsy of ICR mice after the end of observation, abnormal findings were not observed in any of the male mice at all doses (Table S2C and D). The retention of clear fluid in the uterus was observed in female mice in the 50 mg/kg group ( $n=1$ , Table S2D). Thus, these results suggest that melittin-dKLA does not affect the abnormality of clinical signs and necropsy findings in repeated subcutaneous administration in ICR mice in ICR mice and has a lethal dose value higher than 20 mg/kg.

### **Change in relative organ weights after the repeated subcutaneous administration of melittin-dKLA in ICR mice**

To measure the changes in the weight of the organs in ICR mice caused by melittin-dKLA, after subcutaneously administering melittin-dKLA every other day at doses of 0, 1.25, 5, and 20 mg/kg for 4 weeks, the organ tissues of mice were collected and the kidney, thymus, heart, liver, adrenal, spleen, epididymis, testis, prostate, seminal vesicle, ovary, uterus, and lung were weighed. A statistically significant increase in the relative organ weight of the spleen was observed in males in the 20 mg/kg group ( $p < 0.05$ ) (Fig. 3A). In females, a statistically significant difference in relative organ weight measurement was not observed in any of the treatment groups compared to the control group (Fig. 3B).

### **Hematological parameters after the repeated subcutaneous administration of melittin-dKLA in ICR mice**

To evaluate the changes in hematological parameters after the repeated subcutaneous administration of melittin-dKLA in mice, blood samples were collected after the end of the observation period and used to calculate the total erythrocyte count (RBC), RBC distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean platelet volume (MPV), hematocrit (HCT), hemoglobin distribution width (HDW), hemoglobin (HGB), total leukocyte count (WBC), WBC differential count, neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), large unstained cells (LUC), platelets (PLT), and reticulocytes (Retic). As a result,

a statistically significant decrease in MONO was observed in males in the 20 mg/kg group ( $p < 0.05$ ) and a statistically significant increase in NEU was observed in females in the 20 mg/kg group ( $p < 0.05$ ) compared to the control group. In other parameters, there was no significant change by melittin compared to the control group (Fig. 4). The decrease of monocytes can often appear as a result of drug administration, and the increase of neutrophils can occur as a result of acute injection. Thus, these results are the changes observed fall within the expected biological variation, and therefore, it is considered that had no toxicological significance.

### **Clinical chemistry after the repeated subcutaneous administration of melittin-dKLA in ICR mice**

To test the changes in the clinical chemistry of melittin-dKLA in mice, blood samples obtained from mice were analyzed for various parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose (GLU), blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (T-CHO), creatine kinase (CK), total protein (TP), albumin (ALB), total bilirubin (T-BIL), triglycerides (TG), A/G ratio (A/G), and calcium (Ca). As a result, a statistically significant increase in AST was observed in males in the 5 mg/kg group ( $p < 0.05$ ), increase in Ca was observed in males in the 20 mg/kg group ( $p < 0.05$ ), and decrease in BUN, T-BIL, and A/G were observed in males in the 200 mg/kg group ( $p < 0.05$ ) compared to the control group (Fig. 5A–D). Moreover, a statistically significant decrease in T-BIL was observed in females in the 20 mg/kg group ( $p < 0.05$ ) and decrease in A/G was observed in females in the 5 mg/kg and 20 mg/kg groups ( $p < 0.05$ ) compared to the control group (Fig. 5E–H).

## **Discussion**

Melittin as a cytolytic peptide derived from bee venom has been explored in preclinical animal models for various diseases such as arthritis, rheumatism, and chronic pain (Jeong 2015; Lee 2014). Recently, melittin has been widely used as an anti-tumor agent (Zhou et al. 2021). In previous studies, we synthesized a new peptide called Melittin-dKLA, which is synthesized with the pro-apoptotic peptide (dKLA), using the features that melittin binds preferentially to M2 Macrophages, and have reported to have an anti-cancer effect by targeting M2-like TAMs in lung cancer model (Lee et al. 2019). Although melittin has possibility as a therapeutic agent for various diseases, it has been known to induce extensive hemolysis, which can exhibit a severe side effect. The hemolytic activity of melittin has been reported with an IC<sub>50</sub> of  $0.51 \pm 0.12 \mu\text{M}$  (Soman 2009). Furthermore,

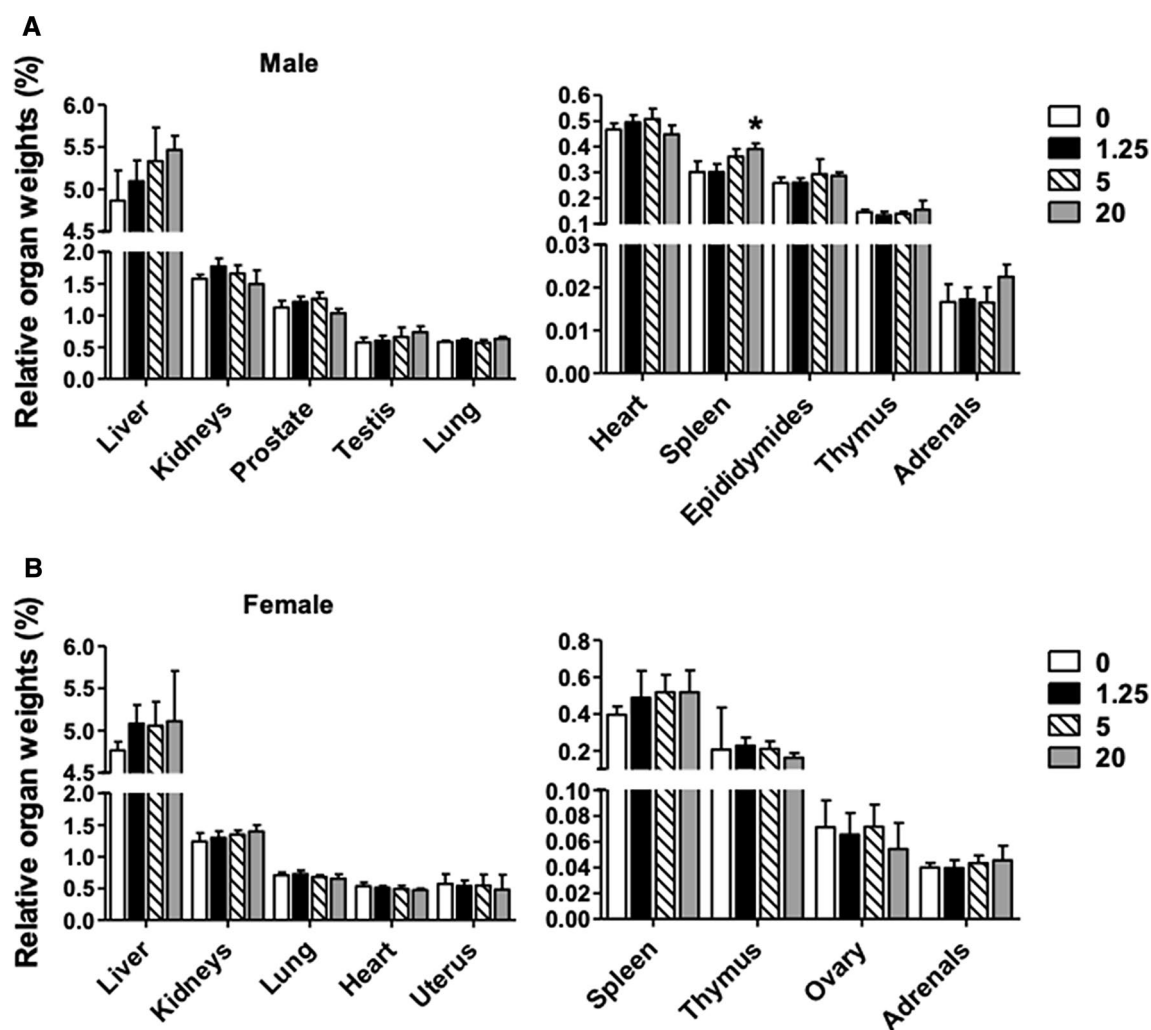


**Fig. 3** Change in relative organ weights in ICR mice after the repeated subcutaneous administration of melittin-dKLA. ICR mice (6 weeks old,  $n=5$ /sex/dose group) were subcutaneously administered melittin-dKLA with doses of 0, 1.25, 5, and 20 mg/kg. For organ weights, the following organs of sacrificed animals were weighed individually: kidney, thymus, heart, liver, adrenal, spleen,

epididymis, testis, prostate, seminal vesicle, ovary, uterus, and lung. Paired organs or seminal vesicle and prostate were weighed together. The relative organ weights of male (A) and female (B) mice were calculated based on fasted body weights. The data shown are the means  $\pm$  SD. \* $p < 0.05$  compared with the control group (0 mg/kg)

many other authors reported cytotoxicity of melittin in various cancer cells (Rady et al. 2017). Also, we confirmed that melittin-dKLA has hemolytic effect ( $IC_{50}$  1.12  $\mu$ M) in red blood cells of mouse (data not shown). However, the limitations of this study did not extend to the in vitro assays of melittin-dKLA. In further studies, an in vitro cytotoxicity study of melittin-dKLA is needed.

Preliminary toxicology studies as the initial phases of development for new substance are usually conducted a dose range finding (DRF) study in rodent (Finch et al. 2019). The first in vivo toxicity study is a common test used for the dose selection, and determined normally by parameters such as clinical signs, body weight changes, food consumption, morbidity and mortality, as well as the hematological and

biochemical analysis for several dose selection (Andrade 2016). Besides that, the single or repeated-dose toxicity studies are performed during the preliminary phase of the development process. The single administration is usually observed for 14 days after treatment, and the repeated-dose administration is evaluated for 28 days (Parasuraman 2011). Particularly, most of the peptide is known to administrate intravenously or subcutaneously due to degradation by Proteases in Gastrointestinal Tract (Bruno et al. 2013), and melittin has reported to have a lethal dose median ( $LD_{50}$ ) of 2.5–5 mg/kg in intravenous injection of the mouse model (Zhou et al. 2021). However, the lethal dose of the subcutaneous injection of melittin in the mouse model is not reported. Thus, the purpose of this study was to evaluate the

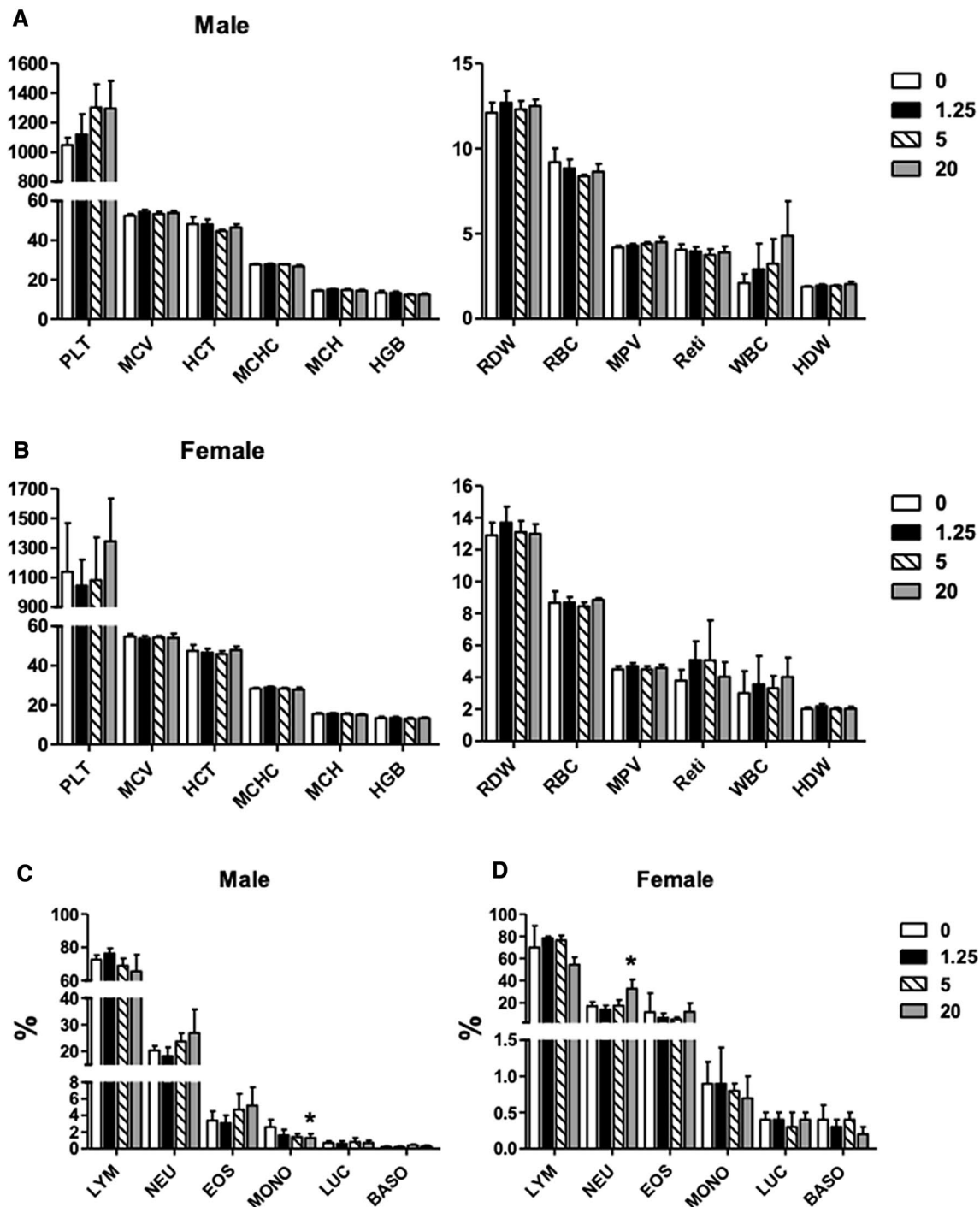


**Fig. 4** Hematological parameters in ICR mice after the repeated subcutaneous administration of melittin-dKLA. ICR mice (6 weeks old,  $n=5$ /sex/dose group) were subcutaneously administered melittin-dKLA with doses of 0, 1.25, 5, and 20 mg/kg. The following parameters of male (A and C) and female (B and D) mice were measured by the nephelometric analysis method with a coagulation time analyzer: total erythrocyte count (RBC), RBC distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin

concentration (MCHC), mean corpuscular hemoglobin (MCH), mean platelet volume (MPV), hematocrit (HCT), hemoglobin distribution width (HDW), hemoglobin (HGB), total leukocyte count (WBC), WBC differential counting; neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), large unstained cells (LUC), platelet (PLT), and reticulocytes (Retic). The data shown are the means  $\pm$  SD. \* $p < 0.05$  compared with the control group (0 mg/kg)

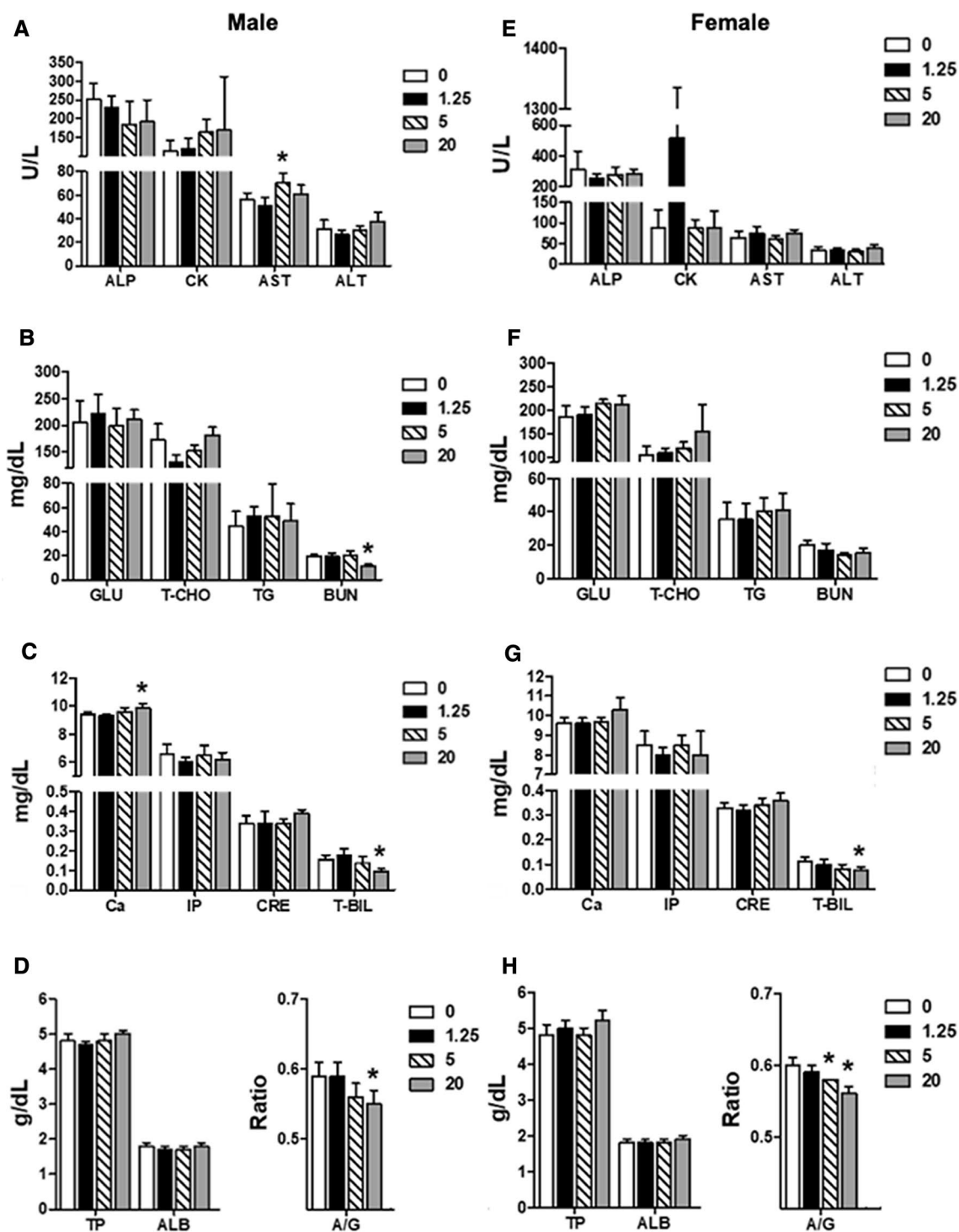


**Fig. 5** Clinical chemistry in ICR mice after the repeated subcutaneous administration of melittin-dKLA. ICR mice (6 weeks old,  $n=5$ /sex/dose group) were subcutaneously administered melittin-dKLA with doses of 0, 1.25, 5, and 20 mg/kg. For clinical chemistry, the following parameters of male (A–D) and female (E–H) mice were analyzed using a blood biochemical analyzer: aspartate aminotrans-

ferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose (GLU), blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (T-CHO), creatine kinase (CK), total protein (TP), albumin (ALB), total bilirubin (T-BIL), triglycerides (TG), A/G ratio (A/G), and calcium (Ca). The data shown are the means  $\pm$  SD. \* $p < 0.05$  compared with the control group (0 mg/kg)

toxicity and the approximate lethal dose values of melittin-dKLA in ICR mice following a single subcutaneous administration and the potential toxicity of melittin-dKLA in ICR mice subcutaneously administered for 4 weeks (every other day). The toxicity profiles of melittin-dKLA were analyzed using several parameters, including mortality, clinical signs, body weight changes, food consumption, hematology, serum biochemistry, necropsy findings, and relative organ weights.

In toxicology analyses, changes in body weight in mice are used to evaluate toxicity doses, and body weight loss (BWL) is one of the few objective measures assessed in toxicity studies (Chapman 2013). The results presented in this study showed recovery after weight loss in the 0, 5, 10, and 20 mg/kg groups of mice subcutaneously administered melittin-dKLA. With regards to weight loss, similar reductions were found in the control group and considered to be temporary symptoms rather than effects of melittin-dKLA. Similarly, after repeated subcutaneous administration in mice, no significant differences between were observed for body weight between the treatment groups and the control group in neither male nor female mice. However, increased food consumption was observed in male and female mice in the 20 mg/kg group compared to the control group. Because mice were fed in group housing and due to the small sample size of this study, it was not possible to conduct statistical analyses. Therefore, we suggest future studies evaluate the significance of the increased food consumption observed in the mice in the 20 mg/kg group using an increasing sample size.

In terms of clinical signs, no abnormalities were observed in mice after a single subcutaneous administration of melittin-dKLA. On the other hand, both male and female mice in the 5 and 20 mg/kg groups showed crust formation and induration after the repeated subcutaneous administration of melittin-dKLA. In both male and female mice, symptoms were observed earlier, but more mice were observed in the 20 mg/kg group than in the 5 mg/kg group. Once a symptom appeared, it was observed continuously until the end of the administration period. Because these symptoms were observed earlier with a higher administration concentration, we expected that the symptoms were caused by the administration of melittin-dKLA.

According to the results of the toxicity analyses, none of the ICR mice died as a result of being subcutaneously administered melittin-dKLA in a single or repeated dose. Therefore, the value of the lethal dose of melittin-dKLA must be above 20 mg/kg.

Exposure to various toxins may cause a variety of hematologic abnormalities, including the dysregulation of the synthesis of cellular elements, a shortened red cell half-life, abnormalities in coagulation, and reduced oxygen-carrying capacity. The toxins found in pharmacologic agents, heavy metals, and venom are associated with the induction of

hematologic abnormalities (Berling et al. 2015). As a result of the repeated subcutaneous administration of melittin-dKLA, the MONO levels of the male mice in the 20 mg/kg group were significantly lower than those of the control group, while the neutrophils (NEU) of the female mice in the 20 mg/kg group were significantly higher than those of the control group, according to the hematological parameters evaluated. Monocytes often decrease as a result of medication or steroid administration, while neutrophils often increase as a result of acute infection. However, the changes observed fall within the expected biological variation (Knoll et al. 1996), and as such had no toxicological significance.

Clinical chemistry analysis revealed that AST significantly increased in male mice in the 5 mg/kg group, while T-BIL significantly decreased in the male and female mice in the 20 mg/kg group. A/G significantly decreased in the female mice in the 5 and 20 mg/kg groups and in the male mice in the 20 mg/kg group. BUN and Ca significantly decreased and increased in male mice in the 20 mg/kg group compared to the control group, respectively. AST, which increased in the male mice in the 5 mg/kg group, is used to screen for liver and liver disease (Mukry 2017). Thus, the content of AST in the blood increases when there is a complication in the liver or myocardium (Knoblauch et al. 2018; Siska et al. 2017; Weingand et al. 1996). However, dose dependency was not observed in this study, implying no significant toxicological implications. BUN is the final decomposed product of protein produced by the urea cycle in the liver and is discharged into the kidneys. Decreased BUN levels have been reported to be the result of severe liver diseases, malnutrition, or nephrotic syndrome (Harr 2002; Seyrek et al. 1996). In this study, the BUN values did not have any toxicological implications, as no changes were observed in any of its related compounds (e.g., creatinine). T-BIL is synthesized from heme isolated when red blood cells are destroyed and can be used to identify the secretory function of the liver (Nakanuma, 2010). Although not always, elevated T-BIL level (hyperbilirubinemia) can cause the abnormal liver function, while low T-BIL level generally does not cause issues (Roy-Chowdhury et al. 2017). Therefore, no toxicological significance was expected. The levels of A/G, which decreased in the female mice in the 5 and 20 mg/kg groups and the male mice in the 20 mg/kg group, is known to decrease as a result of malnutrition (Rahman et al. 2005). In this study, only a slight change in A/G in the treatment groups was observed compared with the control group, indicative of a lack of toxicological significance.

During the necropsy of female mice in the 5 mg/kg group, clear fluid was found to be retained in the uterus. However, this phenomenon can occurs under physiological conditions, depending on the female sexual cycle (proestrus or estrus) (Shimotsuma et al. 2019; Sikora 2016), and is not related to the administration of melittin-dKLA.

In conclusion, the subcutaneous administration of melittin-dKLA in ICR mice, whether single (1 day) or repeated (14 days), did not result in any abnormal signs. No body weight changes, mortality, or clinical signs were found that could be attributed to treatment in any of the groups of experimental animals. Notably, upon the administration of melittin-dKLA in ICR mice every other day for 4 weeks, crust formation and induration were observed in the high dose (20 mg/kg) group. However, no other significant abnormal signs were observed, including body weight changes, mortality, organ weights, and biochemistry. These results suggest that the toxicity of melittin-dKLA administered subcutaneously in ICR mice is higher than 20 mg/kg. Hence, the use of with a high dose of 20 mg/kg and lower doses with an appropriate common ratio appear to be appropriate doses with which to conduct a 13-week repeated toxicity study in the future, and it is suggested that it will be an important data in the development of new drug for melittin-dKLA.

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## Declarations

**Conflict of interest** Author Jiham Sung declares that he has no conflict of interest.

Author Yura Kim declares that she has no conflict of interest.

Author Pei Fu Yu declares that he has no conflict of interest.

Author Younsu Kim declares that he has no conflict of interest.

Author Ik-Hwan Han declares that he has no conflict of interest.

Author Hyunsu Bae declares that he has no conflict of interest.

**Ethical approval** This study was approved by the Institutional Animal Care and Use Committee (IACUC, No. 19M041) in accordance with the “Animal Protection Act” (no. 16075, revised on March 25, 2019).

## References

Alonezi S et al (2017) Metabolomic profiling of the synergistic effects of melittin in combination with cisplatin on ovarian cancer cells. *Metabolites*. <https://doi.org/10.3390/metabo7020014>

- Andrade EL et al (2016) Non-clinical studies required for new drug development - Part I: early in silico and in vitro studies, new target discovery and validation, proof of principles and robustness of animal studies. *Braz J Med Biol Res* 49:e5644. <https://doi.org/10.1590/1414-431X20165644>
- Aufschnaiter A et al (2020) Apitoxin and its components against cancer, neurodegeneration and rheumatoid arthritis: limitations and possibilities. *Toxins (basel)*. <https://doi.org/10.3390/toxins12020066>
- Berling I, Isbister GK (2015) Hematologic effects and complications of snake envenoming. *Transfus Med Rev* 29:82–89. <https://doi.org/10.1016/j.tmr.2014.09.005>
- Bruno BJ, Miller GD, Lim CS (2013) Basics and recent advances in peptide and protein drug delivery. *Ther Deliv* 4:1443–1467. <https://doi.org/10.4155/tde.13.104>
- Carpene M, Nunez-Estevez B, Soria-Lopez A, Simal-Gandara J (2020) Bee venom: an updating review of its bioactive molecules and its health applications. *Nutrients*. <https://doi.org/10.3390/nu12113360>
- Chapman K et al (2013) A global pharmaceutical company initiative: an evidence-based approach to define the upper limit of body weight loss in short term toxicity studies. *Regul Toxicol Pharmacol* 67:27–38. <https://doi.org/10.1016/j.yrtph.2013.04.003>
- Ellerby HM et al (1999) Anti-cancer activity of targeted pro-apoptotic peptides. *Nat Med* 5:1032–1038. <https://doi.org/10.1038/12469>
- Finch SC, Munday JS, Sprosen JM, Bhattarai S (2019) Toxicity studies of chancoclavine in mice. *Toxins (basel)*. <https://doi.org/10.3390/toxins11050249>
- Foillard S et al (2008) Synthesis and biological characterisation of targeted pro-apoptotic peptide. *ChemBioChem* 9:2326–2332. <https://doi.org/10.1002/cbic.200800327>
- Gajski G, Garaj-Vrhovac V (2013) Melittin: a lytic peptide with anticancer properties. *Environ Toxicol Pharmacol* 36:697–705. <https://doi.org/10.1016/j.etap.2013.06.009>
- Harr KE (2002) Clinical chemistry of companion avian species: a review. *Vet Clin Pathol* 31:140–151. <https://doi.org/10.1111/j.1939-165x.2002.tb00295.x>
- Jamasbi E, Mularski A, Separovic F (2016) Model membrane and cell studies of antimicrobial activity of melittin analogues. *Curr Top Med Chem* 16:40–45. <https://doi.org/10.2174/1568026615666150703115919>
- Javadpour MM et al (1996) De novo antimicrobial peptides with low mammalian cell toxicity. *J Med Chem* 39:3107–3113. <https://doi.org/10.1021/jm9509410>
- Jeong YJ et al (2015) Melittin has a chondroprotective effect by inhibiting MMP-1 and MMP-8 expressions via blocking NF-kappaB and AP-1 signaling pathway in chondrocytes. *Int Immunopharmacol* 25:400–405. <https://doi.org/10.1016/j.intimp.2015.02.021>
- Jo M et al (2012) Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway. *Toxicol Appl Pharmacol* 258:72–81. <https://doi.org/10.1016/j.taap.2011.10.009>
- Kim HY et al (2011) The cell penetrating ability of the proapoptotic peptide, KLAKLAKKLAKLAK fused to the N-terminal protein transduction domain of translationally controlled tumor protein MIIYRDLISH. *Biomaterials* 32:5262–5268. <https://doi.org/10.1016/j.biomaterials.2011.03.074>
- Knoblauch SE, Hohl TM, La Perle KMD (2018) Pathology principles and practices for analysis of animal models. *ILAR J* 59:40–50. <https://doi.org/10.1093/ilar/ilz001>
- Knoll JS, Rowell SL (1996) Clinical hematology. In-clinic analysis, quality control, reference values, and system selection. *Vet Clin North Am Small Anim Pract* 26:981–1002. [https://doi.org/10.1016/s0195-5616\(96\)50051-3](https://doi.org/10.1016/s0195-5616(96)50051-3)
- Kwon MK et al (2008) Antitumor effect of a transducible fusogenic peptide releasing multiple proapoptotic peptides by caspase-3.

- Mol Cancer Ther 7:1514–1522. <https://doi.org/10.1158/1535-7163.MCT-07-2009>
- Law B, Quinti L, Choi Y, Weissleder R, Tung CH (2006) A mitochondrial targeted fusion peptide exhibits remarkable cytotoxicity. Mol Cancer Ther 5:1944–1949. <https://doi.org/10.1158/1535-7163.MCT-05-0509>
- Lee G, Bae H (2016) Anti-inflammatory applications of melittin, a major component of bee venom: detailed mechanism of action and adverse effects. Molecules. <https://doi.org/10.3390/molecules21050616>
- Lee JA et al (2014) Bee venom acupuncture for rheumatoid arthritis: a systematic review of randomised clinical trials. BMJ Open 4:e006140. <https://doi.org/10.1136/bmjopen-2014-006140>
- Lee C, Bae SS, Joo H, Bae H (2017) Melittin suppresses tumor progression by regulating tumor-associated macrophages in a Lewis lung carcinoma mouse model. Oncotarget 8:54951–54965. <https://doi.org/10.18632/oncotarget.18627>
- Lee C et al (2019) Targeting of M2-like tumor-associated macrophages with a melittin-based pro-apoptotic peptide. J Immunother Cancer 7:147. <https://doi.org/10.1186/s40425-019-0610-4>
- Lyu C, Fang F, Li B (2019) Anti-tumor effects of melittin and its potential applications in clinic. Curr Protein Pept Sci 20:240–250. <https://doi.org/10.2174/1389203719666180612084615>
- Mai JC, Mi Z, Kim SH, Ng B, Robbins PD (2001) A proapoptotic peptide for the treatment of solid tumors. Cancer Res 61:7709–7712
- Mukry SN et al (2017) Laboratory diagnosis of malaria: comparison of manual and automated diagnostic tests. Can J Infect Dis Med Microbiol 2017:9286392. <https://doi.org/10.1155/2017/9286392>
- Nakanuma Y et al (2010) Application of a new histological staging and grading system for primary biliary cirrhosis to liver biopsy specimens: Interobserver agreement. Pathol Int 60:167–174. <https://doi.org/10.1111/j.1440-1827.2009.02500.x>
- OECD (2008) Test no 407: repeated dose 28-day oral toxicity study in rodents. OECD publishing adopted
- Parasuraman S (2011) Toxicological screening. J Pharmacol Pharmacother 2:74–79. <https://doi.org/10.4103/0976-500X.81895>
- Park MH et al (2011) Anti-cancer effect of bee venom in prostate cancer cells through activation of caspase pathway via inactivation of NF-kappaB. Prostate 71:801–812. <https://doi.org/10.1002/pros.21296>
- Rady I, Siddiqui IA, Rady M, Mukhtar H (2017) Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. Cancer Lett 402:16–31. <https://doi.org/10.1016/j.canlet.2017.05.010>
- Rahman MZ, Begum BA (2005) Serum total protein, albumin and A/G ratio in different grades of protein energy malnutrition. Mymensingh Med J 14:38–40
- Roy-Chowdhury J, Roy-Chowdhury N, Listowsky I, Wolkoff AW (2017) Drug- and drug abuse-associated hyperbilirubinemia: experience with atazanavir. Clin Pharmacol Drug Dev 6:140–146. <https://doi.org/10.1002/cpdd.314>
- Seyrek N, Paydas S, Kilic H, Karayaylali I, Sagliker Y (1996) Nephrotic syndrome accompanying hepatitis-B-related liver cirrhosis and delta hepatitis. Nephron 74:227. <https://doi.org/10.1159/000189311>
- Shimotsu Y, Takeda S, Ogata K, Miyata K (2019) Segmental aplasia of a uterine horn in two RccHan:WIST rats. J Toxicol Pathol 32:293–296. <https://doi.org/10.1293/tox.2019-0015>
- Sikora M et al (2016) The usefulness of uterine lavage and acute phase protein levels as a diagnostic tool for subclinical endometritis in Icelandic mares. Acta Vet Scand 58:50. <https://doi.org/10.1186/s13028-016-0233-4>
- Siska W, Gupta A, Tomlinson L, Tripathi N, von Beust B (2017) Recommendations for clinical pathology data generation, interpretation, and reporting in target animal safety studies for veterinary drug development. Int J Toxicol 36:293–302. <https://doi.org/10.1177/1091581817711876>
- Soman NR et al (2009) Molecularly targeted nanocarriers deliver the cytolytic peptide melittin specifically to tumor cells in mice, reducing tumor growth. J Clin Invest 119:2830–2842. <https://doi.org/10.1172/JCI38842>
- Somwongin S, Chantawannakul P, Chaiyana W (2018) Antioxidant activity and irritation property of venoms from Apis species. Toxicon 145:32–39. <https://doi.org/10.1016/j.toxicon.2018.02.049>
- Therrien A, Fournier A, Lafleur M (2016) Role of the cationic C-terminal segment of melittin on membrane fragmentation. J Phys Chem B 120:3993–4002. <https://doi.org/10.1021/acs.jpcc.5b11705>
- van den Bogaart G, Guzman JV, Mika JT, Poolman B (2008) On the mechanism of pore formation by melittin. J Biol Chem 283:33854–33857. <https://doi.org/10.1074/jbc.M805171200>
- Wang C et al (2009) Melittin, a major component of bee venom, sensitizes human hepatocellular carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by activating CaMKII-TAK1-JNK/p38 and inhibiting I $\kappa$ B $\alpha$  kinase-NF $\kappa$ B. J Biol Chem 284:3804–3813. <https://doi.org/10.1074/jbc.M807191200>
- Weingand K et al (1996) Harmonization of animal clinical pathology testing in toxicity and safety studies. The joint scientific committee for international harmonization of clinical pathology testing. Fundam Appl Toxicol 29:198–201
- Zhou J et al (2021) Delivery strategies for melittin-based cancer therapy. ACS Appl Mater Interfaces 13:17158–17173. <https://doi.org/10.1021/acsami.1c03640>

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