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Original Article

Impact of different blood sampling techniques on plasma biomarkers for skeletal myopathy in conscious rats

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ABSTRACT — To characterize variability of various musculoskeletal biomarkers by different blood sampling techniques in conscious rats, plasma asparate aminotransferase (AST), creatine kinase (CK) and its isoenzymes, fatty acid binding protein 3 (FABP3), myosin light chain 3 (Myl3) and microRNA (miR-133a) obtained by jugular venipuncture (C-JV) or tail venipuncture (C-TV) were compared with those obtained by jugular venipuncture (A-JV) in isoflurane-anesthetized rats. Plasma CK, FABP3 and Myl3, especially when collected by C-TV, were higher with larger variability than when collected by A-JV, whereas miR-133a displayed large variability in all techniques. Interestingly, higher CK obtained by C-JV or C-TV was largely attributable to higher CK-MM or CK-BB, respectively. Handling and restraint stress were identified as possible factors contributing to larger variability for CK, FABP3 and Myl3. A close correlation between CK and FABP3 was demonstrated both in the C-JV and C-TV techniques. Next, we evaluated the impact of C-JV and C-TV techniques for detecting skeletal myopathy in 2,3,5,6-tetramethyl-p-phenylenediamine-treated rats. In this model, CK and CK-MM obtained by C-TV were significantly increased, but those obtained by C-JV were not modified. In contrast, AST, FABP3, Myl3 and miR-133a obtained by both techniques were drastically elevated to a similar extent. The results suggest that, in conscious rats, the tail venipuncture technique may be more appropriate to detect skeletal myopathy despite the higher variability with this technique than with the jugular venipuncture technique. Furthermore, FABP3, Myl3 and miR-133a may serve as more sensitive biomarkers with large signal-to-noise ratios regardless of the blood sampling technique in conscious rats.

Key words: Handling techniques, Plasma biomarkers, Skeletal myopathy, Variability

INTRODUCTION

Blood sampling from laboratory animals is one of the most common procedures in the toxicological studies to assess the potential toxicity of new chemical entities (Morton *et al*., 1993; Diehl *et al*., 2001). However, techniques for blood sampling from small laboratory animals such as rats have different impacts on various factors due to differences in habituation (Goicoechea *et al*., 2008), handling (Goicoechea *et al*., 2008; Kawahara *et al*., 1999; Yerroum *et al*., 1999), restraint (Tabata *et al*., 1998; Vahl *et al*., 2005), bleeding sites (van Herck *et al*., 2001; Matsuzawa *et al*., 1993; Conybeare *et al*., 1988), or anesthesia (Altholtz *et al*., 2006; Arnold and Langhans, 2010; Fitzner *et al*., 2006). Consequently, the different impacts of blood sampling techniques on blood analysis may introduce confounding variables in data interpretation.

Asparate aminotransferase (AST; EC 2.6.1.1) and creatine kinase (CK: EC 2.7.3.2) have been widely used for identifying myopathic adverse effects. CK is a dimeric protein made up of two distinct polypeptide subunits, M (muscle type) and B (brain type). CK has three isoenzymes; skeletal-muscle type CK-MM, cardiac-muscle type CK-MB and brain-type CK-BB. Since CK is present in significant concentrations in skeletal and cardiac muscles and to a lesser extent in gastrointestinal tract and brain, analysis of these CK isoenzymes provides more specific information about injured tissue (Bais and Edward, 1982; Wallimann *et al*., 1992). In addition,

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CK is present physiologically in blood, which indicates it is constitutively released from muscle cells. Importantly, the electrophoretic pattern of CK isoenzymes in plasma or serum has been reported to differ among species; the main isoenzyme in humans (Boone *et al*., 1980) and dogs (Aktas *et al*., 1993) is CK-MM, whereas that in rats (Shibata and Kobayashi, 1978) is CK-BB. Furthermore, Shibata and Kobayashi (1978) suggested that blood platelets possibly contribute to the CK activity of circulating plasma as well as to the activity in serum in rats. Therefore, the fluctuation of these isoenzymes in rats must be interpreted with caution, depending upon the factors which influence both the total and isoenzyme-specific activities. Furthermore, unlike AST, CK has been reported to vary from 100 U/L to 1,300 U/L even in naive rats due to different sampling techniques aforementioned (Goicoechea *et al*., 2008; Yerroum *et al*., 1999; Matsuzawa *et al*., 1993). However, in the majority of toxicological studies, analytical specifications and recommendations are described either poorly or not at all, making it difficult to standardize them for non-terminal repetitive blood sampling (Goicoechea *et al*., 2008). It is important to note that the effects of drugs inducing mild muscular toxicity could possibly be masked as a result of failing to control such factors of variability (Goicoechea *et al*., 2008).

Recently, fatty acid binding protein 3 (FABP3) and myosin light chain 3 (Myl3) have been introduced into the toxicological field as promising biomarkers for skeletal myopathy (Pritt *et al*., 2008; Tonomura *et al*., 2009, 2012). Additionally, muscle specific microRNAs (e.g., miR-133a) have also been suggested as useful and reliable biomarkers for skeletal muscle injury in experimental animals (Laterza *et al*., 2009; Mizuno *et al*., 2011). However, the variability of these new biomarkers associated with different sampling techniques in conscious rats has not been fully elucidated.

To clarify the variability of various musculoskeletal biomarkers by different sampling techniques in conscious male F344 rats, plasma AST, CK and its isoenzymes, FABP3, Myl3 and miR-133a from blood samples obtained by jugular venipuncture (C-JV) or tail venipuncture (C-TV) which are widely used for repeated blood samplings in the toxicological studies, were compared with those obtained by jugular venipuncture (A-JV) in isoflurane-anesthetized rats. Next, the effects of handling, restraint including duration of sampling time, and frequency of needle venipuncture on these biomarkers were examined in conscious rats. Lastly, to confirm whether the variability for these biomarkers in conscious rats have an impact on toxicological evaluation, rats were treated with the representative muscle toxicant, 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) (Munday *et al*., 1990; Dare *et al*., 2002) and the fluctuation of these biomarkers were evaluated by the C-JV and C-TV techniques.

MATERIALS AND METHODS

Animals and housing

One hundred and eighty, specified pathogen-free, male F344 rats were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals in the present study were between 9 and 12 weeks old and weighed between 165 and 266 g. Rats were housed individually in stainless steel wire mesh bracket cages for at least 5 days as acclimation to the laboratory environment. During the study, the animal room was maintained at a temperature of 23 ± 3°C and humidity of 55 ± 15%, and the lighting was on a 12-hr light (from 07:00 to 19:00) and 12-hr dark cycle. Commercial rodent diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were available *ad libitum*. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd. (Tokyo, Japan). All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science (1987).

Blood collection

The purpose of this study was to characterize variability of various musculoskeletal biomarkers in plasma collected by the C-JV and C-TV techniques. These sampling techniques in conscious rats were reported to be suitable for obtaining a relatively large volume of blood and for repeated blood sampling because both methods cause little injury to the whole body without unnecessary blood loss (Diehl *et al*., 2001; Furuhama and Onodera, 1983) and are routinely used in the toxicological studies at our laboratories. To minimize the variability due to blood sampling techniques including handling, restraint, and venipuncture, all experiments were performed by well-trained staff with more than five years of routine experience with the techniques. Furthermore, the animals were habituated to the experimental procedures such as handling, restraint in a restrainer, and jugular or tail venipuncture during a period of at least one week before initiation of each experiment because habituation of animals to laboratory handling procedures was reported to reduce the variability of CK (Goicoechea *et al*., 2008).

A-JV technique

Animals were placed in an induction chamber connect-

ed to a small animal anesthetizer (MK-A100, Muromachi Kikai Co., Ltd., Tokyo, Japan) to induce anesthesia with 4% isoflurane (Mylan Seiyaku Ltd., Pittsburgh, PA, USA) in oxygen. Subsequently, a nose cone was placed on the rats to maintain anesthesia (4% isoflurane in O_2) during the blood collection. After making an incision in the skin, a 25G wing-type needle attached to a 1-mL syringe was passed through the pectoral muscle below the sternoclavicular junction into the jugular vein. Approximately 1 mL of blood was withdrawn from the jugular vein of each rat. After blood collection, the animals were euthanized humanely by exsanguination.

C-JV technique

Animals were held behind the front legs as usually recommended and the fur around the site of insertion of the needle was wiped gently with 70% (v/v) ethanol. A 25G needle attached to 1-mL syringe was passed through the pectoral muscle below the sternoclavicular junction into the jugular vein as was case with the A-JV technique. A slight negative pressure was applied to the syringe until venipuncture was achieved. Approximately 300 to 600 µL of blood was collected on each occasion (within 10% of the circulating blood volume in 24 hr) in reference to the guideline for the recommended maximum blood sampling volume for rats (Diehl *et al*., 2001). Then the needle was withdrawn and digital pressure was applied to the puncture site for a short time to minimize hemorrhage. The duration of sampling time from the beginning of animal handling to the end of blood collection was within 1 min. After the final blood collection, the animals were euthanized humanely by exsanguination.

C-TV technique

Animals were placed in a hand-made restrainer and a 23G wing-type needle equipped with an extension tube (SV-23DLK, Terumo Corporation, Tokyo, Japan) was inserted into the lateral tail vein. Approximately 300 to 600 µL of a natural efflux of blood was collected on each occasion (within 10% of the circulating blood volume in 24 hr). When an adequate volume of blood was collected, the needle was withdrawn and the puncture site was gently pressed with the fingers for a short time. The duration of sampling time from the beginning of animal restraint to the end of blood collection was within 3 min except for the experiment to investigate factors contributing to the variability of biomarkers in this technique. After the final blood collection, the animals were euthanized humanely by exsanguination.

Sample preparation

The obtained whole blood was immediately placed in blood collection tubes containing heparin lithium (CAPIJECT®, Terumo Corporation) or tubes containing EDTA-2K (MICROTAINER®, Japan Becton Dickinson Company, Ltd., Tokyo, Japan). Plasma samples were obtained by centrifugation at 3,000 rpm and 4°C for 10 min with a refrigerated centrifuge (CF7D2, Hitachi Koki Co., Ltd., Tokyo, Japan) and then stored at -80°C until analysis. Heparinized plasma was used for blood chemistry and corticosterone measurement and EDTA plasma was used for microRNA analysis.

Experimental protocols

Variability of plasma musculoskeletal biomarkers in conscious rats

To clarify the variability of various musculoskeletal biomarkers in conscious rats, plasma AST, CK and its isoenzymes, FABP3, Myl3 and miR-133a from blood samples collected by C-JV or C-TV were compared with those collected by A-JV.

Factors contributing to variability in conscious rats

Plasma corticosterone concentrations from blood samples collected by the C-JV and C-TV were compared to examine the stress of each procedure on the animals as previously described (Vahl *et al*., 2005). Moreover, the effects of the number of needle venipuncture in C-JV technique or the duration of the sampling time in C-TV technique on plasma CK, FABP3, Myl3 and miR-133a were examined.

Effect of TMPD treatment in conscious rats

A single dose of TMPD (Sigma, St. Louis, MO, USA) at a dose level of 3 or 9 mg/kg was orally administered to rats. As a vehicle control, 0.5% methylcellulose solution (#400, Nacalai Tesque, Inc., Tokyo, Japan) was used. The dose levels of TMPD were selected based on the previous reports (Munday *et al*., 1990; Dare *et al*., 2002). Blood was collected by C-JV or C-TV just before dosing (designated as 0 hr), and 4, 7, and 24 hr after dosing in the same manner as described above. After the final blood collection, animals were euthanized humanely by exsanguination under isoflurane-anesthesia and a skeletal muscle (right rectus femoris) was excised for pathological examination.

Analysis of plasma musculoskeletal biomarkers and corticosterone

Blood chemistry

Plasma AST and CK activities were measured by an

automatic analyzer (TBA-200FR, Toshiba Medical Co., Ltd., Tokyo, Japan). CK isoenzymes (CK-BB, CK-MB, and CK-MM) were electrophoretically separated using a rapid-type electrophoresis device (REP8JF71000, Helena Laboratories, Saitama, Japan) and the relative percentage of each isoenzyme was calculated. The absolute activity of each isoenzyme was determined from the total CK activity and relative percentage of each isoenzyme. Analyses of plasma FABP3 and Myl3 were carried out using a commercially available Muscle Injury Panel 1 (Meso Scale Discovery, Gaithersburg, MD, USA) and MSD Sector Imager 6000 (Meso Scale Discovery). The detection limits of FABP3 and Myl3 by this platform were 0.391 and 0.781 ng/mL, respectively.

Measurement of miR-133a

Total RNA including miRNA was extracted from 60 to 100 μL of plasma using miRNeasy Mini Kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were frozen at -80°C until use. Total RNA was reverse transcribed at 16°C for 30 min, 43°C for 30 min, and 85°C for 5 min using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and miRNA-specific stem-loop primers (TaqMan miRNA assay kit, Applied Biosystems) in a GeneAmp® PCR System 9700 (Applied Biosystems). The cDNA was stored at -20°C until further use. The target miRNA (miR-133a) was amplified using TaqMan Fast Advanced MasterMix and TaqMan MicroRNA Assays (Applied Biosystems) on an Applied Biosystems 7900HT Fast Real Time PCR System (Applied Biosystems). The thermal cycling conditions were 50°C for 2 min, then 95°C for 20 sec, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec. The expression level of miR-133a was normalized to spiked-in miR-238 (Syn-cel-miR-238 miScript miRNA Mimic, QIAGEN) as an external control. The values of miR-133a are presented as a ratio of the expression level to the mean expression level of the concurrent control group in each experiment.

Measurement of corticosterone

Plasma corticosterone concentrations were determined using a commercially available ELISA kit (AssayMax corticosterone ELISA kit, ASSAYPRO, St. Charles, MO, USA) according to the manufacturer's instructions.

Histopathology

The skeletal muscle specimens were fixed in 10% neutral buffered formalin, and embedded in paraffin, and sections were prepared, stained with hematoxylin and eosin (H&E) and subjected to microscopic examination.

Statistical analysis

Quantitative data are expressed as mean ± S.E. unless otherwise stated. They were statistically analyzed by the Student's t-test, Tukey's multiple comparison test, or Dunnett's multiple comparison test. The relationship between biomarkers was evaluated by Spearman's correlation coefficient statistical analysis. These statistical analyses were performed by the use of SAS System Release 8.2 (SAS Institute Inc., Cary, NC, USA). A P value less than 5% was considered statistically significant. In addition, descriptive statistics incorporating visual descriptions of the variability of musculoskeletal biomarkers were prepared using a box-and-whisker plot method. The lower hinge, median, and upper hinge of the box corresponded to the 25%, 50%, and 75% percentiles, respectively. The length of the box is the inter-quartile range (IQR). The lengths of the whiskers extending from the hinges are defined as 1.5 × IQR unless minimum or maximum value has been reached when outliers are excluded. Outliers were also shown as separate points outside the whiskers which were located between 1.5 × IQR from the hinges.

RESULTS

Variability of plasma musculoskeletal biomarkers in conscious rats

The variability of plasma AST in the C-JV or C-TV group was comparable to that in the A-JV group. On the other hand, plasma CK, FABP3 or Myl3 in the C-JV and C-TV groups showed relatively higher variability than those in the A-JV group. As results, mean values of CK, FABP3 and Myl3 in the C-JV and C-TV groups tended to be higher or were significantly higher than those in the A-JV group. When mean values were compared between the C-JV and C-TV groups, the values of CK and Myl3 were significantly higher in the C-TV group than in the C-JV group. Plasma miR-133a showed large variability in all 3 groups, and no statistically significant differences in mean values were noted among them (Fig. 1).

Electrophoretic analysis of CK isoenzymes revealed that main isoenzyme in plasma obtained by A-JV was CK-BB. Plasma CK-MM in the C-JV group was significantly higher than those in the A-JV and C-TV groups, resulting in higher total CK in this technique. Plasma CK-BB in the C-TV group was significantly higher than those in the A-JV and C-JV groups, resulting in higher total CK in this technique. Plasma CK-MB were different among the three groups, but the differences were very small (Fig. 2).

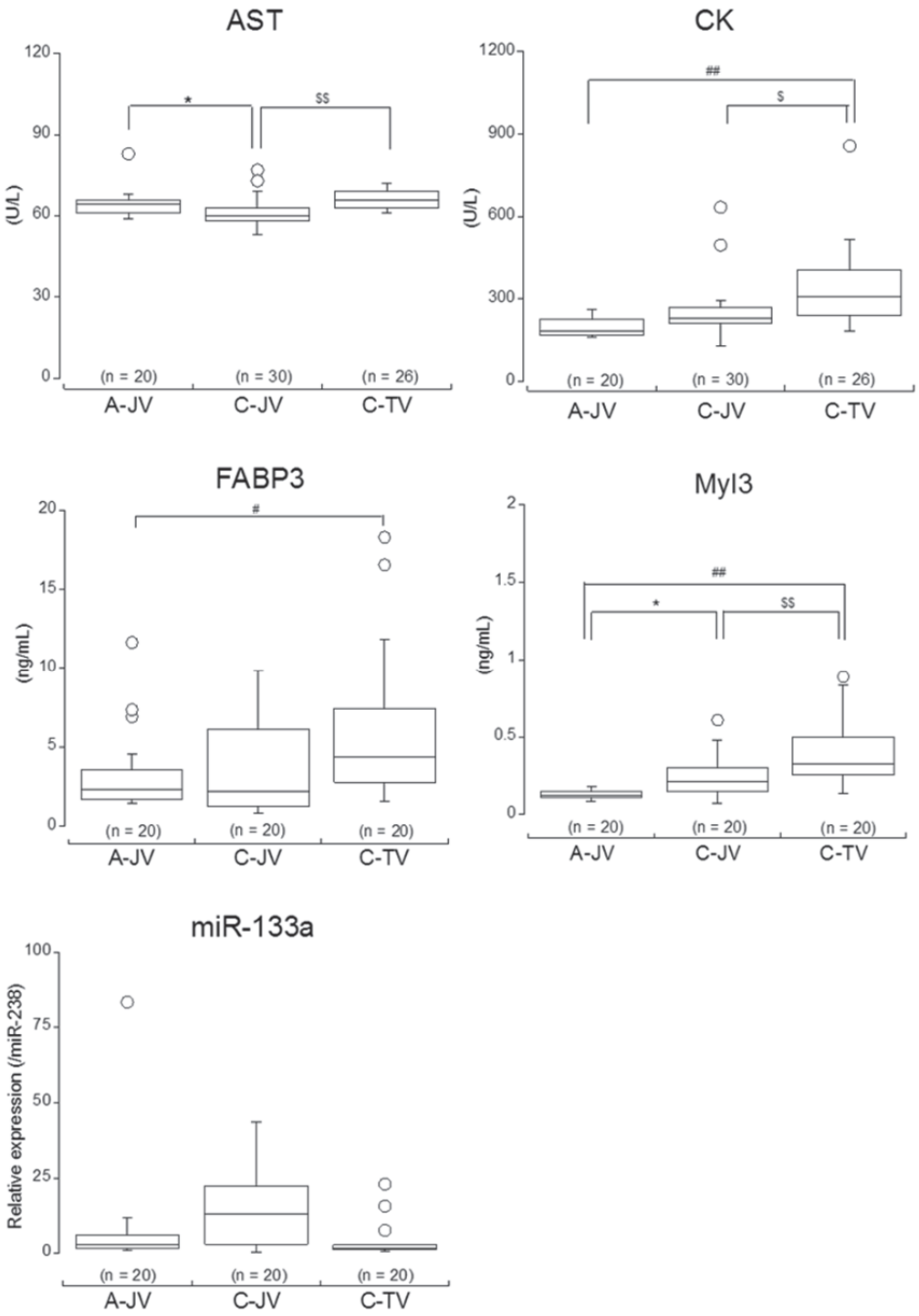

Fig. 1. Variability of plasma AST, CK, FABP3, Myl3, and miR-133a obtained by different sampling techniques; A-JV in isoflurane-anesthetized rats, and C-JV and C-TV in conscious rats. The box-and-whisker plots represent the minimum, 25th percentile, median, 75th percentile, maximum, and outliers. The values in parenthesis represent the number of animals in each group. *P < 0.05: Significantly different from the A-JV group, #P < 0.05, ##P < 0.01: Significantly different from the A-JV group, $P < 0.05, $$P < 0.01: Significantly different from the C-JV group (Tukey's test).

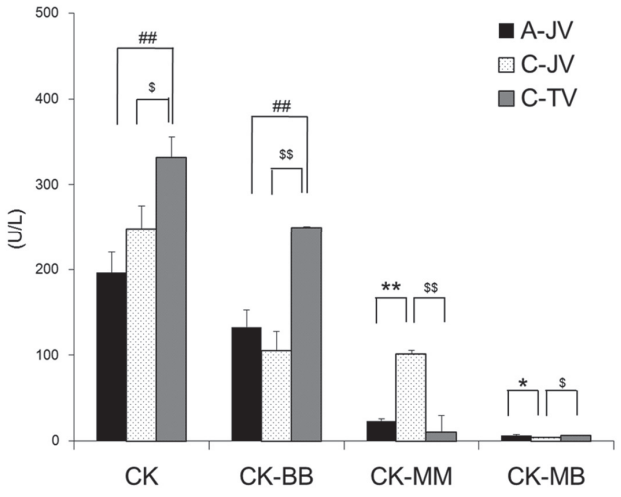

Fig. 2. Variability of plasma total CK and CK isoenzymes obtained by different sampling techniques; A-JV in isoflurane-anesthetized rats, and C-JV and C-TV in conscious rats. Each column and bar represent the mean ± S.E. of 15 to 20 animals. *P < 0.05, **P < 0.01: Significantly different from the A-JV group, ##P < 0.01: Significantly different from the A-JV group, $P < 0.05, $$P < 0.01: Significantly different from the C-JV group (Tukey's test).

Factors contributing to variability in conscious rats

Plasma corticosterone concentrations in the C-TV group were approximately 5 times higher than those in the C-JV group (Fig. 3A). A close correlation was detected between plasma corticosterone and CK obtained from the same blood samples in both techniques ($r = 0.622$, $P < 0.01$, $n = 20$, Fig. 3B). Plasma CK, FABP3, Myl3 and miR-133a in the C-JV group were not affected by the number of needle punctures repeatedly up to 3 times (data not shown). Plasma CK and FABP3 in the C-TV group were substantially higher with a wide variability once the duration of the sampling time exceeded 3 min, whereas plasma Myl3 and miR-133a were not increased by the duration of sampling time (Fig. 3C).

A close correlation was detected between plasma CK and FABP3 obtained from the same blood samples in both C-JV and C-TV techniques. The correlation coefficient was relatively higher in the C-TV group ($r = 0.892$, $P < 0.01$, $n = 60$, Fig. 4B) than that in the C-JV group ($r = 0.675$, $P < 0.01$, $n = 50$, Fig. 4A). No trend was identified between CK and either Myl3 or miR-133a obtained from the same blood samples in both techniques (Figs. 4A and 4B). In addition, no correlation was noted among FABP3, Myl3 and miR-133a obtained from the same blood samples in both techniques (data not shown).

Effect of TMPD treatment in conscious rats

No pathological abnormalities were noted in the skeletal muscle of any rat given vehicle or 3 mg/kg of TMPD. Skeletal muscle myopathy, which was characterized by vacuolation and degeneration of myocytes, was observed 24 hr post-dose in all the animals given 9 mg/kg of TMPD.

In the C-JV group, plasma AST was significantly increased from 4 hr post-dose in rats given TMPD at 9 mg/kg, and plasma AST at 24 hr post-dose was 5 times higher than that of the vehicle control group (Fig. 5A). In contrast, plasma total CK and CK-MM were not different from those of the vehicle control group at any time point (Fig. 5A). In addition, plasma CK-BB and CK-MB in rats given TMPD at 9 mg/kg were comparable to those of the vehicle control group at any time point (data not shown). Plasma FABP3, Myl3 and miR-133a started to be increased significantly from 7 hr post-dose in rats given TMPD at 9 mg/kg (Fig. 6A). At 24 hr after administration of TMPD at 9 mg/kg, the mean values of plasma FABP3, Myl3 and miR-133a were approximately 10, 80, and 15 times, respectively, higher than those of the vehicle control group (Fig. 6A).

In the C-TV group, plasma AST and CK-MM started to be increased significantly from 7 hr post-dose, and plasma CK was significantly increased 24 hr post-dose in rats given TMPD at 9 mg/kg (Fig. 5B). At 24 hr after administration of TMPD at 9 mg/kg, the mean values of plasma AST, CK and CK-MM were significantly higher by 6, 2 and 20 times, respectively, compared to those of the vehicle control group (Fig. 5B). Plasma CK-BB and CK-MB in rats given TMPD at 9 mg/kg were comparable to those of the vehicle control group at any time point (data not shown). Plasma FABP3, Myl3 and miR-133a started to be increased from 7 hr post-dose in rats given TMPD at 9 mg/kg (Fig. 6B). The mean values of FABP3 and miR-133a at 7 hr after dosing of TMPD at 9 mg/kg were approximately 8 and 4 times higher than those of the vehicle control group, but they were not significantly different because of large variability. At 24 hr after administration of TMPD at 9 mg/kg, the mean values of FABP3, Myl3 and miR-133a were significantly higher by 13, 20, and 20 times compared to those of the vehicle control group (Fig. 6B).

DISCUSSION

It is crucial to understand variability of biomarkers due to sampling techniques and to minimize it for iden-

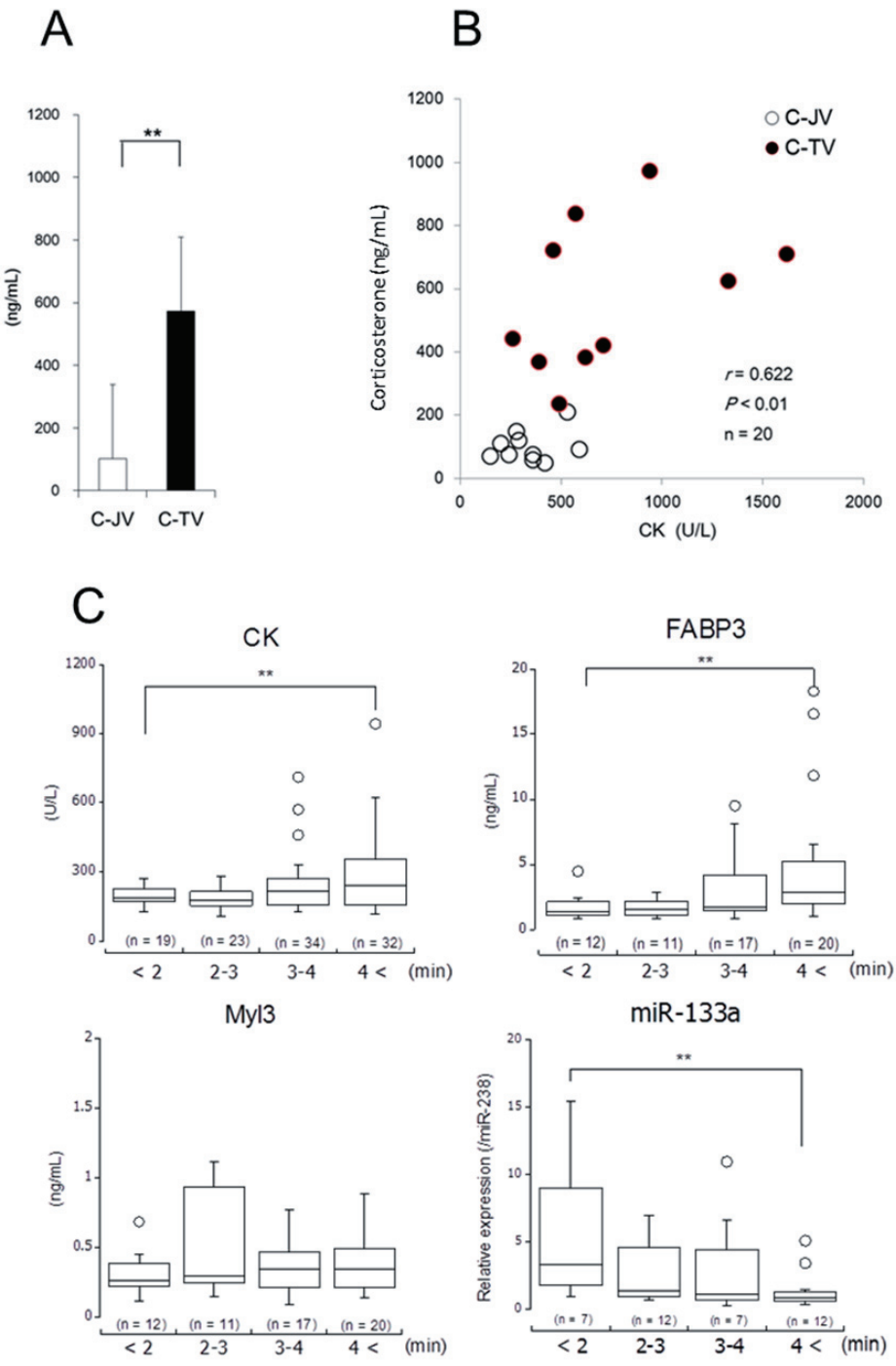

Fig. 3. Factors contributing to variability of plasma musculoskeletal biomarkers. (A) Plasma corticosterone concentrations. Each column and bar represent the mean ± S.E. of 10 animals of each technique. **P < 0.01: Significantly different from the C-JV group (Student's t-test), (B) Correlation between plasma corticosterone and CK obtained from the same blood samples, Open circle; samples obtained by C-JV, Solid circle; samples obtained by C-TV, (C) Duration of sampling time in C-TV technique. The box-and-whisker plots for the duration of sampling time represent the minimum, 25th percentile, median, 75th percentile, maximum, and outliers. The values in parenthesis represent the number of animals in each group. **P < 0.01: Significantly different from the group with sampling time less than 2 min (Dunnett's test).

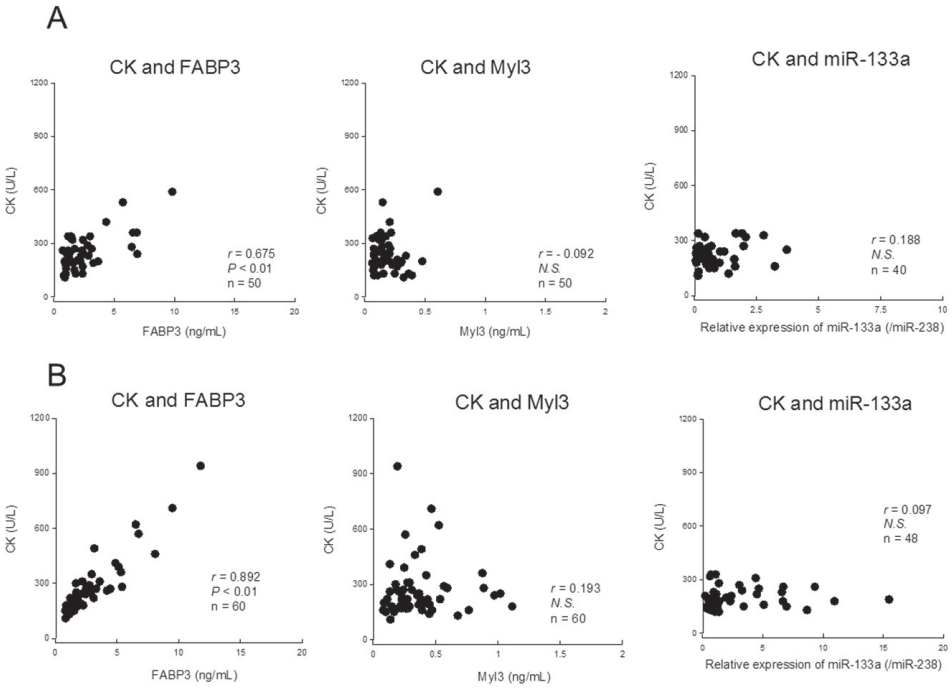

Fig. 4. Relationship between CK and FABP3, Myl3 or miR-133a collected by different sampling techniques in conscious rats; (A) Jugular venipuncture technique, (B) Tail venipuncture technique. N.S.: Not statistically significant.

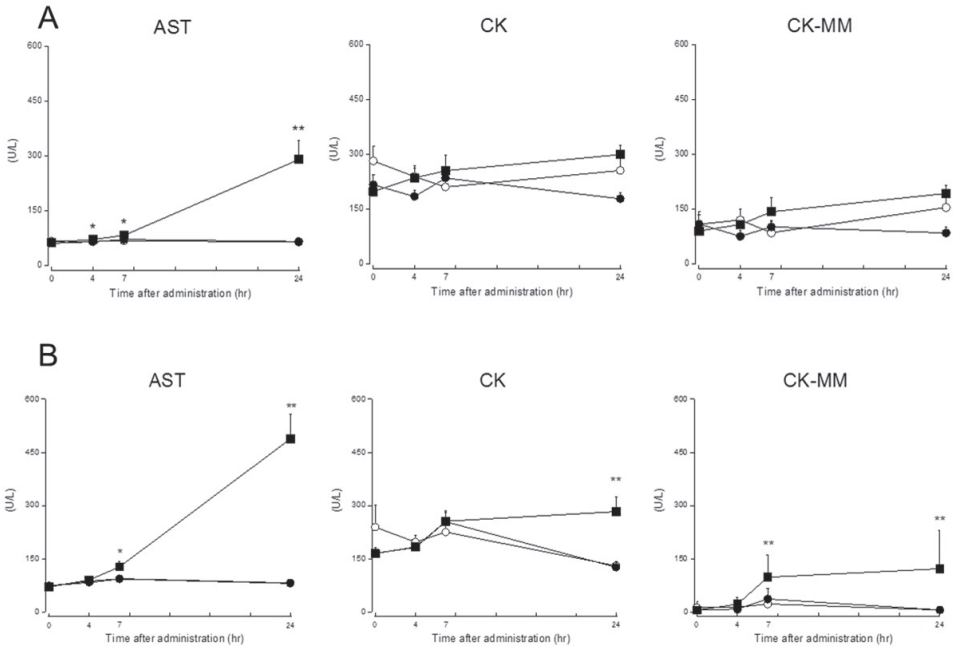

Fig. 5. Effect of TMPD on plasma AST, CK and CK-MM collected by different sampling techniques in conscious rats; (A) Jugular venipuncture technique, (B) Tail venipuncture technique; Open circle; vehicle control group, Solid circle; TMPD 3 mg/kg group, Solid square; TMPD 9 mg/kg group. Each circle and bar represent the mean ± S.E. of 6 to 10 animals. *P < 0.05, **P < 0.01: Significantly different from the vehicle control group (Dunnett's test).

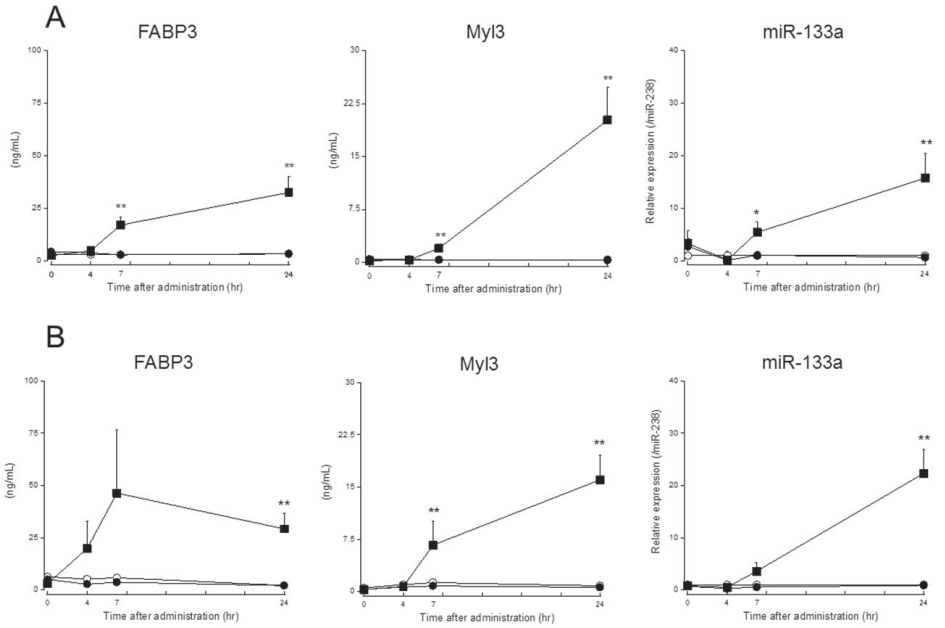

Fig. 6. Effect of TMPD on plasma FABP3, Myl3, and miR-133a collected by different sampling techniques in conscious rats; (A) Jugular venipuncture technique, (B) Tail venipuncture technique; Open circle; vehicle control group, Solid circle; TMPD 3 mg/kg group, Solid square; TMPD 9 mg/kg group. Each circle and bar represent the mean ± S.E. of 6 to 10 animals. *P < 0.05, **P < 0.01: Significantly different from the vehicle control group (Dunnett's test).

tifying drug-induced toxic signals correctly since different blood sampling techniques may introduce confounding variables in data interpretation. To address this issue, we characterized the variability of various musculoskeletal biomarkers by using different sampling techniques and the potential factors contributing to the variability in conscious male F344 rats.

In the present study, the C-JV or C-TV technique had negligible effect on variability of plasma AST compared to the A-JV technique. On the other hand, plasma CK obtained either by C-JV or C-TV was higher with a wider variability compared to that obtained by A-JV. Electrophoretic analysis of CK isoenzymes revealed that the main isoenzyme in plasma obtained by A-JV was CK-BB, which is consistent with the previous reports (Shibata and Kobayashi, 1978). Interestingly, blood collection from different sites in conscious rats exhibited a substantial influence on CK isoenzyme ratios, a finding which has not been previously reported. Namely, the higher total CK obtained by C-JV was largely attributable to higher CK-MM, whereas the higher total CK obtained by C-TV was due mainly to higher CK-BB. These results suggest that the C-JV technique may provide more confounding variability of CK isoenzyme that is specific to skeletal myopathy than the C-TV technique even though the latter increased the total CK with a larger variability than the former. We also found similar influences of blood sampling techniques on variability of plasma FABP3 and Myl3 in conscious rats. In contrast, plasma miR-133a had large variability in all three techniques, resulting in no statistically significant difference in the mean values of miR-133a among them. Comparing the variability of these biomarkers between C-JV and C-TV techniques, the C-TV technique showed relatively higher values with larger variability than the C-JV technique.

Stress by habituation (Goicoechea *et al*., 2008), handling (Goicoechea *et al*., 2008; Kawahara *et al*., 1999; Yerroum *et al*., 1999), and restraint (Tabata *et al*., 1998; Vahl *et al*., 2005) has been suggested to contribute to large variability of various biomarkers such as CK. Even though rats in our study were accustomed to laboratory handling procedures for more than one week to reduce the variability due to the habituation and handling procedures according to the report by Goicoechea *et al*. (2008), CK collected by C-JV exhibited relatively higher variability than that collected by A-JV. Likewise, stress

from the sampling procedure has been reported to cause mild to moderate release of FABP3 into blood (O'Brien, 2008). Moreover, restraint stress during blood sampling may cause more discomfort to animals (Tabata *et al*., 1998; Vahl *et al*., 2005). In fact, higher plasma corticosterone concentrations were noted in the C-TV group than in the C-JV group and a close correlation between plasma corticosterone and CK was identified. Furthermore, CK and FABP3 values in the C-TV group were considerably high with large variability once the duration of sampling time exceeded 3 min at which time the basal corticosterone levels were elevated through the initiation of the pituitary stress response (Vahl *et al*., 2005), indicating that the higher values with large variability of CK and FABP3 were attributable to the stress by the restraint. Intriguingly, a close correlation between plasma CK and FABP3 obtained from the same blood samples was identified not only in the C-TV group but also in the C-JV group, suggesting that plasma CK and FABP3 might be subject to influence by the handling and restraint stress to a similar extent in conscious rats. Meanwhile, it is important to note that the extended blood sampling time could activate coagulation parameters because no anticoagulant was added to the syringe or extension tube for blood sampling in the present study. This was, at least in part, supported by the fact that the higher total CK in the C-TV group was mainly attributable to higher CK-BB as this enzyme was only found in platelets (Shibata and Kobayashi, 1978). Furthermore, it could not be denied that hemolysis might affect these enzyme activities, since leakage from erythrocytes results in elevated CK (Matsuzawa *et al*., 1993), though no obvious evidence of hemolysis was detected in plasma collected by C-TV. A variety of techniques are available for obtaining an adequate volume of blood from the tail vein of conscious rats in a short time without blood coagulation and hemolysis during blood sampling. These techniques include nicking or incising the tail (Conybeare *et al*., 1988; Fluttert *et al*., 2000) and inserting the needle into the vein when the environment or the tail itself is heated up (Vahl *et al*., 2005; Furuhama and Onodera, 1983). Although our preliminary experiment demonstrated that plasma CK obtained by C-TV without anticoagulant was comparable to that collected by the C-TV with heparin, it is important to examine whether the above alternative techniques could minimize the confounding variability of CK and FABP3 due to the restraint stress. Little is known regarding influence of blood sampling procedures on Myl3 and miR-133a to our knowledge. Plasma Myl3 was higher and had a wider variability in both the C-JV and C-TV techniques than in the A-JV technique, indicating that the stress by sampling procedure including handling and restraint might cause mild to moderate release of Myl3 into blood as was case with CK and FABP3. On the other hand, neither the duration of sampling time nor the number of needle venipunctures had an impact on the variability of Myl3 or miR-133a. Moreover, no trend was identified between plasma CK and either Myl3 or miR-133a in either technique. These results imply that the factors contributing to variability of Myl3 and miR-133a might be different from those of CK or FABP3. However, further investigation is needed to clarify the factors contributing to variability of these biomarkers.

The confounding variability due to blood sampling techniques aforementioned may result in false-negative conclusion. To evaluate this possibility, we examined the fluctuations of various musculoskeletal biomarkers collected by C-JV and C-TV in a TMPD-induced skeletal muscle injury model. Treatment with TMPD at 9 mg/kg induced myopathy characterized by vacuolation and degeneration of myocytes in the skeletal muscles of all the animals at 24 hr post-dose. Plasma CK-MM and total CK in the C-TV group were increased significantly from 7 hr and 24 hr after the TMPD treatment, but plasma CK-MM and total CK in the C-JV group did not change even at 24 hr after the TMPD treatment. These results clearly demonstrated that the C-TV technique may be appropriate method than the C-JV technique to detect skeletal myopathy, even though the C-TV technique has some disadvantages including large variability and troublesome procedures. In contrast, plasma AST, FABP3, Myl3 and miR-133a were drastically elevated after TMPD treatment and the extent of the change was similar in both techniques. The large signal-to-noise ratios seen with these biomarkers regardless of sampling technique indicate their diagnostic accuracy in line with the previous work (Pritt *et al*., 2008; Tonomura *et al*., 2009; Tonomura *et al*., 2012; Laterza *et al*., 2009). The kinetics of these biomarkers were somewhat different; plasma CK-MM and FABP3 were drastically elevated 7 hr after the TMPD treatment, whereas plasma AST, Myl3 and miR-133 reached maximum at 24 hr after the TMPD treatment. Thus, it is critical that investigators should consider the kinetics of each biomarker response to various types of compounds depending on their mode of action when interpreting results of the studies. Overall, combinational measurement of these biomarkers could supply useful information for detecting potential toxicity and target tissues for new chemical entities in nonclinical toxicological studies as several authors pointed out (Pritt *et al*., 2008; Tonomura *et al*., 2009; Tonomura *et al*., 2012).

The present results provide important information on the confounding variability of various musculoskeletal biomarkers due to different blood sampling techniques in conscious rats. The tail venipuncture technique in conscious rats may be more appropriate method to detect skeletal myopathy despite the higher variability with this technique than with the jugular venipuncture technique. Furthermore, FABP3, Myl3 and miR-133a may serve as more sensitive biomarkers with large signal-to-noise ratios than those of the conventional biomarkers like CK even in conscious rats. Our findings emphasize the need to select the blood sampling site and collection method considering their advantages and disadvantages where repetitive blood samples are required.

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

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