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Ultrasonographic measurement of the renal resistive index in the cynomolgus monkey (*Macaca fascicularis*) under conscious and ketamine-immobilized conditions

Hiroya KONNO, Tomomichi ISHIZAKA, Katsuyoshi CHIBA and Kazuhiko MORI

Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd., 1-16-13 Kita-Kasai, Edogawa-ku, Tokyo 134-8630, Japan

Abstract: Measurement of the renal resistive index (RRI) is one of the standard diagnostic procedures for assessing kidney disability clinically. This method is expected to be used for the same purpose in many kinds of animals, including monkeys utilized in conventional toxicology studies. To establish a practical RRI measurement procedure in cynomolgus monkeys (*Macaca fascicularis*), RRI was measured by ultrasonography in the spine position in conscious and ketamine-immobilized monkeys. The RRI of conscious monkeys and ketamine-immobilized monkeys could be measured consistently without excessive abdominal or thoracic movement. Consequently, the variability of the RRI in conscious monkeys was comparable to that in ketamine-anesthetized monkeys. No sex difference in RRI was noted between the two conditions. The mean values and SD of the RRI of 48 healthy monkeys (n=24/sex) were 0.55 ± 0.07 and 0.50 ± 0.05, under conscious and ketamine-immobilized conditions, respectively. The RRI of ketamine-immobilized monkeys was significantly lower than that of conscious monkeys, correlating with the decreased blood pressure and heart rate. In a monkey model of cisplatin-induced acute renal injury, which was characterized histopathologically by minimal to mild renal tubular necrosis and regeneration, the RRI was increased beyond the cut off value (mean + 2SD, 0.68) associated with the progression of renal pathogenesis. The present results suggest that ultrasonographic measurement of the RRI in conscious monkeys would be a useful tool in conventional toxicology studies evaluating drug-induced renal injury.
Key words: cisplatin, non-human primate, renal ultrasonography

Introduction

The diagnosis of renal disorders clinically involves the performance of numerous investigations including ultrasound duplex, computed tomography angiography, magnetic resonance angiography, and catheterization [7]. Among these diagnostic procedures, the duplex method specialized in imaging the vasculature is the gold standard procedure because it allows for noninvasive evaluation of real-time anatomical and dynamic data related to the kidney [22]. In the duplex method, the renal resistive index (RRI), which is calculated from the pulse wave of the blood flow velocity, is known to be not only higher in patients with renal artery disease, including renal artery stenosis and renal aneurysms [8], but also in those with acute and chronic renal failure [3, 9]. In addition, the RRI was reported to be increased in dogs [15] and cats [23] with renal disease. Recently, it has been reported that the RRI was increased by renal injuries caused by angiotensin inhibitors [13] and contrast media [27] in patients and cisplatin [5] in rats. Therefore, RRI is accepted as an early diagnostic biomarker for drug-induced renal injury.

In non-clinical safety assessment for pharmaceuticals, toxicity studies in rodents and non-rodents are warranted in accordance with the "Guidance on the Conduct of Non-clinical Studies for Application for Marketing and Clinical Trials of Drugs (ICH M3)" [11]. The non-clinical guideline for pharmaceuticals (ICH S6 guideline) states that it is important to select relevant animal species for toxicity testing due to the species specificity

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Corresponding author: H. Konno. e-mail: konno.hiroya.e5@daiichisankyo.co.jp

of many biotechnology-derived pharmaceuticals [12]; monkeys are selected as a relevant animal species in many cases [25]. The cynomolgus monkey (*Macaca fascicularis*) is one of the most commonly used animals for preclinical toxicity evaluation because, as non-human primates, monkeys are considered to be anatomically, physiologically, and genetically closer to humans than any other laboratory animals, including rats and mice [19, 24]. Despite the importance of the usage of cynomolgus monkeys in non-clinical toxicity testing, only one report is available regarding the RRI measurement of normal monkeys under ketamine-immobilized and isoflurane-anaesthetized conditions [6]. However, ketamine and/or isoflurane has been reported to affect RRI [6, 16]. Therefore, in nonclinical toxicity testing, RRI measurement of conscious monkeys is more appropriate for the assessment of drug candidates to avoid the effects of chemical immobilization and anesthesia.

The present study was designed to establish a practical RRI measurement procedure in cynomolgus monkeys that can be applied in conventional toxicology studies. In the present study, the RRIs of 48 healthy monkeys were measured using ultrasonography under conscious and ketamine-immobilized conditions. Furthermore, the time course of change of the RRIs in a monkey model of cisplatin-induced acute renal injury was investigated to confirm the utility of RRI as a quantitative biomarker of renal injury.

Materials and Methods

Drugs

Cisplatin (Randa Inj.) was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Ketamine hydrochloride (KETALAR®) was purchased from Daiichi Sankyo Co., Ltd. (Tokyo, Japan).

Animals

A total of 48 cynomolgus monkeys (23 males and 19 females of Cambodian origin, 1 male and 3 females of Vietnamese origin, 1 female of Chinese origin, and 1 female of Indonesian origin) were obtained from Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan), Hamri Co., Ltd. (Ibaraki, Japan), or CLEA Japan, Inc. (Tokyo, Japan). At the receipt, all the animals were approximately 2 years of age. After the arrival of monkeys in our facility, animals were habituated routinely by handling and restraint every 2 weeks until use. At the initiation of the present study, the animals were between 3 and 13 years of age and weighed between 2.45 and 5.95 kg. The conditions of the animals were confirmed to be healthy by veterinarian before the initiation of the present study. During the habituation and study periods, the monkeys were housed individually or paired in stainless steel cages (W 594 mm × D 870 mm × H 1,015 mm) in an animal study room maintained at a temperature of 24°C and humidity of 60%. The housing was maintained with a 12-h light/dark cycle (lights on from 07:00 to 19:00). Commercial pellets for monkeys (PS-A, Oriental Yeast Co., Ltd., Tokyo, Japan) were given to each animal (100 g/day) in the morning (from 7:00 to 10:00), except for the days of RRI measurement and dosing, and tap water was available *ad libitum*.

Experimental protocol

This study was conducted in compliance with the "Law Concerning the Protection and Control of Animals" (Japanese Law No. 105, October 1, 1973) and "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Organizations under the jurisdiction of the Ministry of Health Labour and Welfare" (Notification No. 0601001, issued by the Japanese Ministry of Health Labour and Welfare, dated June 1, 2006). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd. All animal procedures were performed in accordance with the guideline of the Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. The study was divided into the following two experiments: Experiment 1 (measurements of RRI, systolic blood pressure [SBP], mean blood pressure [MBP], diastolic blood pressure [DBP], and heart rate [HR]) and Experiment 2 (assessment of the effect of cisplatin on RRI). To minimize the effect of physical movement on RRI measurements, all experiments were performed by well-trained staff with more than 5 years of experience with routine experiments. Furthermore, the animals were habituated to the experimental procedure, such as handling and restraint, during a period of at least 1 week before the initiation because restraint in a monkey chair was reported to increase both abdominal and thoracic movements in conscious monkeys [6].

Experiment 1: Measurement of RRI, SBP, MBP, DBP, and HR in conscious and ketamine-immobilized monkeys

Animals were captured in the supine position and their necks were restrained using an immobilization device while keeping their hands and legs opened (Fig. 1). First, the RRI was measured while the monkey was conscious. On the day following RRI measurement under the conscious condition, animals were immobilized using an intramuscular injection of ketamine hydrochloride at 10 mg/kg. The dose level of ketamine hydrochloride was

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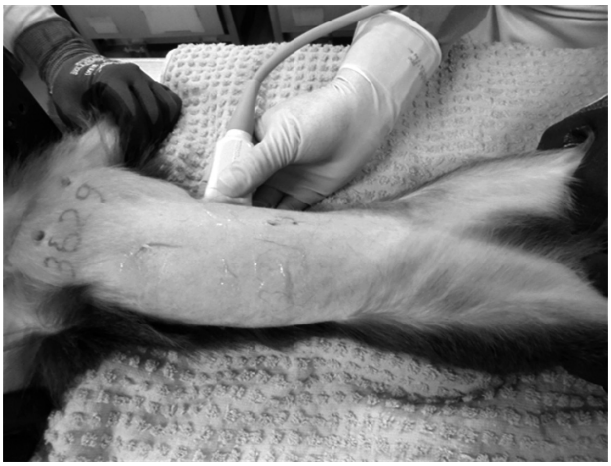

Fig. 1. The method of animal restraint for the measurement of renal resistive index (RRI) in cynomolgus monkeys. A monkey was restrained in the supine position and its neck was restrained with an immobilization device while keeping their hands and legs open.

set to immobilize cynomolgus monkeys reliably [28]. As it was practically possible to measure the RRI of 9 to 10 animals per day, the 48 animals (24 animals/sex) were separated into five groups (9–10 animals/group). SBP, MBP, DBP, and HR were also measured in four groups (19 animals/sex, 38 animals in total).

Experiment 2: Effect of cisplatin on RRI

A single dose of cisplatin at 2.5 mg/kg was administered intravenously to three male monkeys. The dose was selected to induce kidney injury in cynomolgus monkeys based on a previous report [2]. The dosing day was designated as Day 1. In the study of the time course of changes in RRI, ultrasonography was performed before dosing (Day −15 and Day −8), 2 to 4 h after dosing, and Day 3 and Day 7 in conscious monkeys. Approximately 0.6 ml of blood was also collected from the femoral vein to measure plasma creatinine (CRE) and urea nitrogen (UN) levels after each RRI measurement. All the animals were euthanized humanely under anesthesia with an intravenous injection of pentobarbital sodium (25 mg/kg, Somnopentyl Injection, Kyoritsu Seiyaku Co., Tokyo Japan) for pathological examination of the kidneys after the final RRI measurement on Day 7.

Ultrasonography

After shaving the abdomen of the animals with a hair clipper, a convex-type probe with ultrasound diagnostic gel (Sonojery, Canon Medical Supply Co., Ltd., Tokyo, Japan) was placed on the abdomen. A long-axis cross-section including the renal pelvis of the left kidney was drawn using an ultrasound diagnostic instrument (NemioMX, Canon Medical Systems Co., Ltd., Tochigi, Japan). Renal blood flow rates were measured with conditions selected for which kidney images, color Doppler, and blood flow waveforms were most clearly delineated for each individual (frequency range: 7.0 to 8.0 Hz, color gain: 2 to 17 Hz, and Doppler gain: 4 to 22 Hz). After confirming the position of blood flow in the renal intraparenchymal artery using a color Doppler, the Doppler radar angle was adjusted so that the angle of incidence with respect to the blood vessel was visually 60 degrees or less, according to the guideline of methods for evaluating renal artery lesions clinically [22]. During the measurement, the image was stopped after three waveforms were observed with clear systolic peak blood flow rate (PSV) and end diastolic blood flow velocity (EDV) (excluding those with clear abnormal waveforms due to body motion and respiration) within the monitor. The RRI was then calculated as [PSV-EDV] divided by PSV and the RRIs of the three waveforms were averaged to obtain an individual value.

Measurement of SBP, MBP, DBP, and HR

SBP, MBP, DBP, and HR were measured in the spine position with the cuff (Size: 4–8 cm or 6–11 cm) placed on the tail using an animal sphygmomanometer (BP100D, Fukuda ME Kogyo Co., LTD., Tokyo, Japan). All parameters were measured three times and the median was recorded.

Measurement of plasma CRE and UN

The collected blood was injected into tubes (MICROTAINER®, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing heparin lithium, and plasma was obtained following centrifugation at 4°C and 3,000 rpm for 10 min. Plasma CRE and UN levels were measured using an automated analyzer (TBA-2000FR, Canon Medical Systems Co., Ltd.).

Histopathology

The left and right kidneys were fixed in 10% neutral buffered formalin, embedded in paraffin, stained with hematoxylin and eosin, and examined microscopically.

Statistical analysis

In Experiment 1, mean, SD, 2SD, and coefficient of variation (CV) of RRI, SBP, MBP, DBP, and HR were calculated separately under conscious and ketamine-immobilized conditions. Comparisons of the measurement values between conditions were analyzed by a paired t-test. In addition, sex and age differences in each parameter were analyzed using an F-test to evaluate the homogeneity of variance. The parameters were further analyzed using a Student's *t*-test for homogenous data

and an Aspin-Welch's *t*-test for non-homogenous data. The relationship between RRI and SBP, MBP, and DBP or HR was evaluated using Pearson's correlation coefficient analysis. Furthermore, the relationship of these examination items between conditions in each animal was evaluated using Pearson's correlation coefficient analysis. In Experiment 2, quantitative data are expressed as a mean ± SD. They were statistically analyzed by a paired *t*-test to compare post-dose values with pre-dose values for each parameter. These statistical analyses were performed using SAS® System Release 9. 2 (SAS Institute Inc., Cary, NC, USA). A *P* value less than 5% was considered statistically significant.

Results

RRI, SBP, MBP, DBP, and HR in conscious and ketamine-immobilized monkeys

Typical ultrasonography of the kidney of monkeys is shown in Fig. 2. The variability of RRI in conscious monkeys was comparable to that of ketamine-immobilized monkeys (Table 1). Furthermore, no sex differences were observed in RRI, SBD, MBP, DBP, or HR under both conditions, except for DBP and HR values under the ketamine-immobilized condition (Table 1). Furthermore, no correlation was observed between RRI and age under both conditions in the present study (Fig. 3). The mean values of RRI, SBP, MBP, DBP, and HR in both sexes in the conscious condition were statistically higher than those in the ketamine-immobilized condition (Table 1). In addition, close correlations were detected between RRI and SBP, MBP, DBP, and HR in the ketamine-immobilized condition (Fig. 4). The RRI in each animal between conditions was not correlated, whereas SBP, MBP, DBP, and HR between conditions were statistically correlated (Fig. 5).

Fluctuation of RRI in monkey cisplatin-induced renal injury model

Single intravenous administration of cisplatin at 2.5 mg/kg caused minimal to mild renal tubular necrosis and regeneration in all animals (Fig. 6, Table 2). Furthermore, inflammation accompanied by mononuclear cell infiltration was noted in the interstitium of two males (Animal Nos. 1 and 3). Hyaline casts were also noted in Animal No. 1 (Table 2). In these monkey models of cisplatin-induced renal injury, the RRIs were not changed

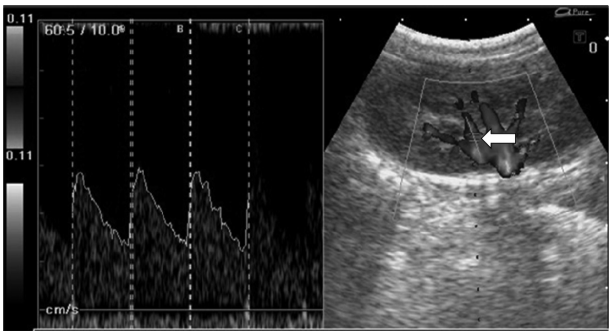

Fig. 2. Typical ultrasonography of the kidney using the pulse and color oppler mode of the duplex ultrasound in cynomolgus monkeys. Ultrasonography was performed using NemioMX. An arrow indicates the measurement site of renal blood flow in the renal segmental or interlobar artery.

Table 1. Renal resistive index, and systolic, mean, and diastolic blood pressure, and heart rate in cynomolgus monkeys under conscious or ketamine-immobilized conditions

Items	Sex	N	Conscious condition				Ketamine-immobilized condition			
			Mean	SD	2SD	CV	Mean	SD	2SD	CV
Renal resistive index	Male	24	0.56	0.07	0.14	0.12	0.51	0.05	0.09	0.09
	Female	24	0.54	0.07	0.13	0.12	0.50	0.06	0.12	0.13
	All	48	0.55	0.07	0.14	0.12	0.50*	0.05	0.10	0.11
Systolic blood pressure	Male	19	131.8	19.7	39.4	0.15	116.9	16.6	33.1	0.14
	Female	19	131.5	17.0	34.0	0.13	120.2	20.5	40.9	0.17
	All	38	131.7	18.1	36.3	0.14	118.5*	18.4	36.9	0.16
Mean blood pressure	Male	19	95.5	14.6	29.3	0.15	81.6	13.3	26.7	0.16
	Female	19	98.0	16.2	32.4	0.17	88.5	15.5	31.1	0.18
	All	38	96.7	15.3	30.6	0.16	85.1*	14.7	29.4	0.17
Diastolic blood pressure	Male	19	77.0	13.3	26.6	0.17	64.4	12.5	25.0	0.19
	Female	19	81.4	15.9	31.8	0.20	72.5#	13.7	27.5	0.19
	All	38	79.2	14.6	29.2	0.18	68.4*	13.6	27.2	0.20
Heart rate	Male	19	223.2	18.1	36.2	0.08	168.3	25.9	51.7	0.15
	Female	19	229.4	28.8	57.5	0.13	195.3#	25.9	51.7	0.13
	All	38	226.3	23.9	47.8	0.11	181.8*	29.0	57.9	0.16

CV: Coefficient of variation. *P<0.05: Significantly different from the conscious condition (paired *t*-test). #P<0.05: Significantly different between males and females (Student's *t*-test).

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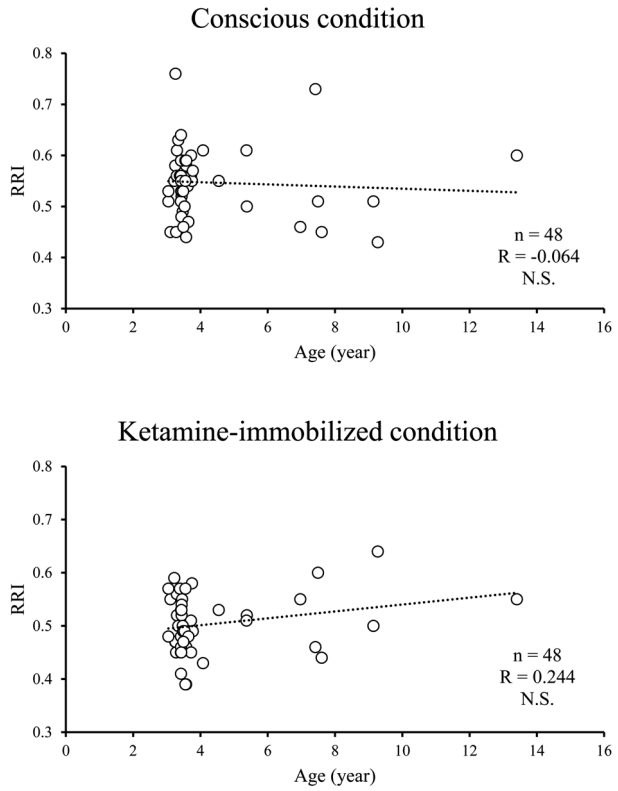

Fig. 3. Relationship between renal resistive index and age in cynomolgus monkeys under conscious and ketamine-immobilized conditions. RRI: renal resistive index, R: Pearson's correlation coefficient, N.S.: not statistically significant

until Day 3. These values were significantly increased on Day 7. Slight increases in CRE and UN levels were noted in two males (Animal Nos. 1 and 3) on Day 3 or later, but the mean values of three males were not statistically significant (Table 3).

Discussion

Gaschen *et al.* reported that abdominal and thoracic movement associated with respiration in conscious monkeys affected RRI measurement and therefore chemical restraint by immobilization or anesthesia was practically useful for measurement [6]. Conversely, chemical immobilization and anesthesia were reported to affect RRI [6, 16]. Furthermore, clinical ultrasonography of the abdominal organs, including the kidneys, is practically performed with patients in the supine position [10]. In monkeys, the supine position is also considered suitable for keeping the animal in position in contrast to the sitting position. Therefore, in the present study, RRI measurements were carried out with the monkeys in the supine position in conscious and ketamine-immobilized conditions. Consequently, the RRI measurement of monkeys in the supine position in the conscious condition could be measured consistently as well as in the ketamine-immobilized condition, without excessive abdominal and thoracic movement associated with respiration.

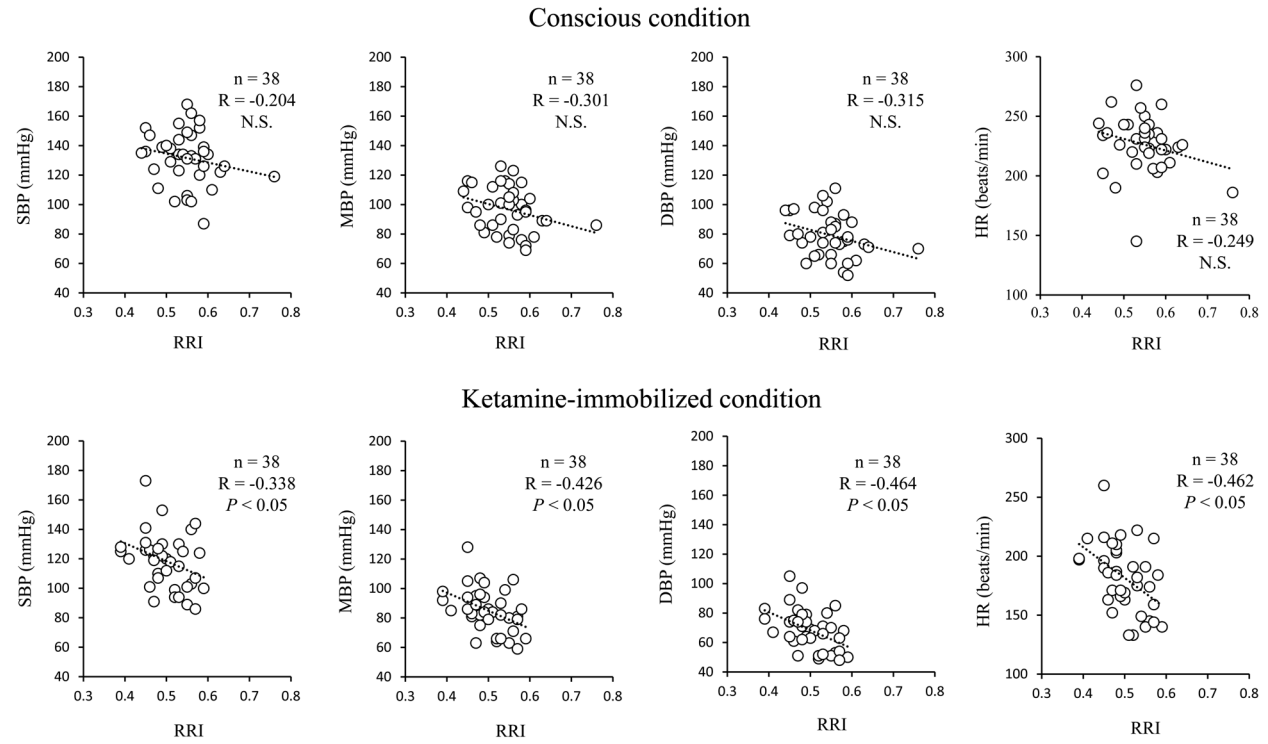

Fig. 4. Relationship between renal resistive index and systolic, mean, and diastolic blood pressure or heart rate in cynomolgus monkeys under conscious and ketamine-immobilized conditions. RRI: renal resistive index, SBP: systolic blood pressure, MBP: mean blood pressure, DBP: diastolic blood pressure, HR: heart rate, R: Pearson's correlation coefficient, N.S.: not statistically significant

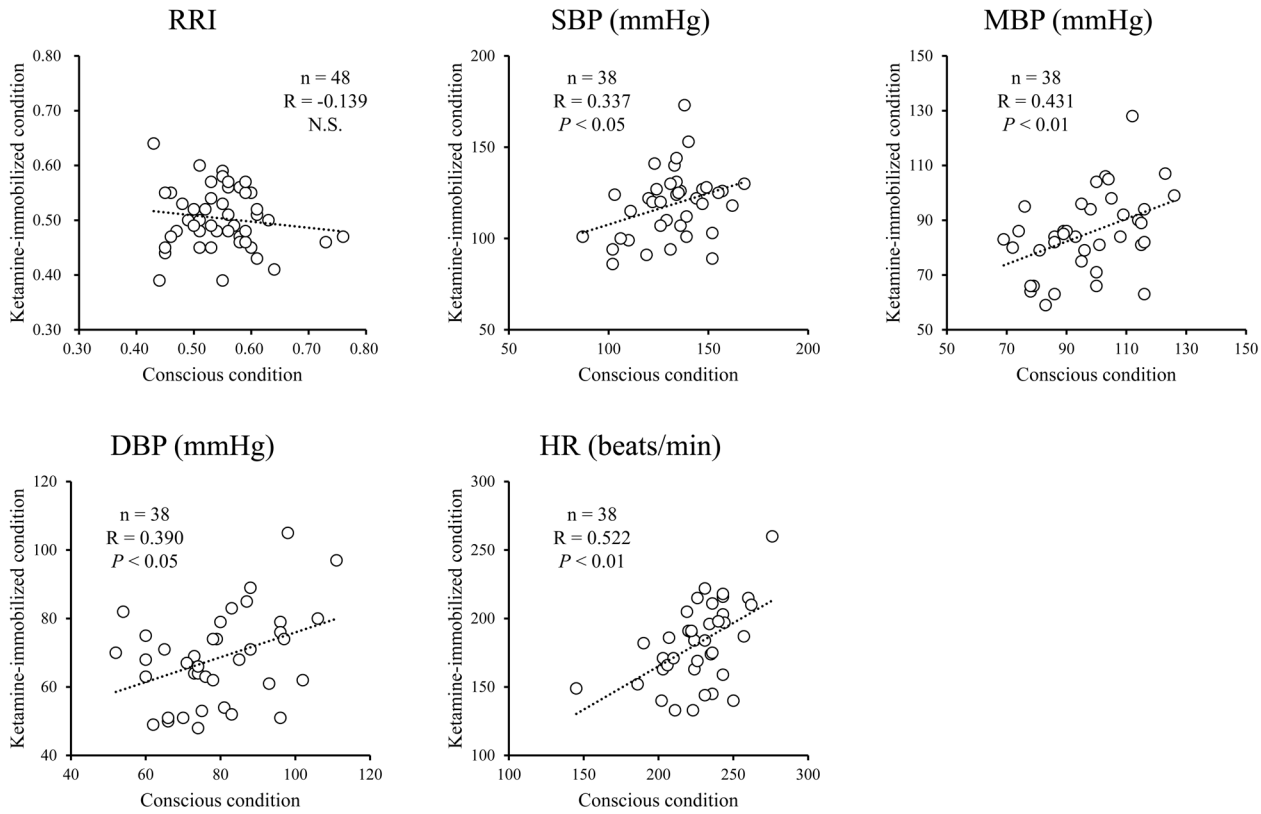

Fig. 5. Relationship of renal resistive index, and systolic, mean, and diastolic blood pressure or heart rate between conscious and ketamine-immobilized conditions in each cynomolgus monkey. RRI: renal resistive index, SBP: systolic blood pressure, MBP: mean blood pressure, DBP: diastolic blood pressure, HR: heart rate, R: Pearson's correlation coefficient, N.S.: not statistically significant

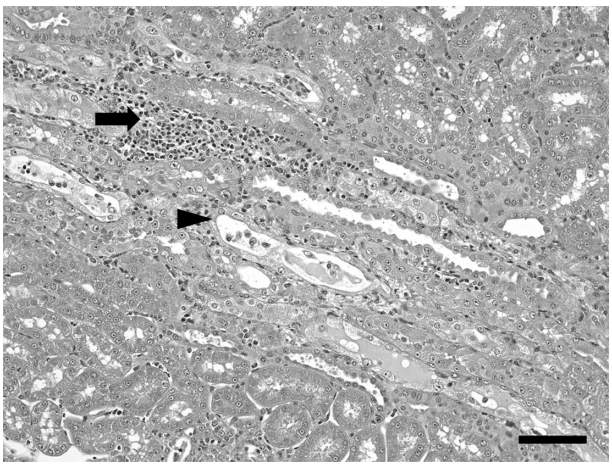

Fig. 6. Representative hematoxylin and eosin image of the kidney in cynomolgus monkey (Animal No.1) treated with cisplatin. Mild necrosis and regeneration of renal tubular (arrowhead) with mononuclear cell infiltration in the interstitium (arrow) were observed. Bar indicates 100 μm.

Table 2. Cisplatin-induced renal injuries in cynomolgus monkeys

Animal number	Histopathological findings in the kidneys
No.1	Regeneration, renal tubule (2) Necrosis, renal tubule (2) Inflammation, mononuclear cell, interstitium (2) Cast, hyaline (2)
No.2	Regeneration, renal tubule (2) Necrosis, renal tubule (1)
No.3	Regeneration, renal tubule (2) Necrosis, renal tubule (1) Inflammation, mononuclear cell, interstitium (2)

Histopathological grades are as follows: 1, minimal; 2, mild.

The RRIs of monkeys in the conscious condition were significantly higher than those in the ketamine-immobilized condition. It is known that ketamine increases renal blood flow at lower doses and decreases it at higher doses [16]. The RRI (0.54) of ketamine-immobilized monkeys reported by Gaschen *et al.* [6] was slightly higher than that in our study (0.50), but rather was comparable to that in conscious monkeys in our study (0.55). Although the cause of the difference in RRI values in the ketamine-immobilized condition between our study and the previous one [6] remains unresolved, a relatively higher dose of ketamine used in this study (10 mg/kg) compared to their study (10 mg/body) might decrease RRI in our study compared to the previous study by Gaschen *et al.* [6]. This was supported by the fact that decreased RRI in the ketamine-immobilized condition was correlated with decreases in systemic blood pressure and heart rate. It has also been reported that high-dose

Table 3. Fluctuation of renal resistive index and plasma urea nitrogen and creatinine levels in cynomolgus monkeys treated with cisplatin

Parameter	Animal number	Days			
		Pre	1	3	7
Renal resistive index	No.1	0.57	0.46	0.48	0.66
	No.2	0.54	0.55	0.45	0.71
	No.3	0.51	0.42	0.64	0.69
	Mean	0.54	0.48	0.52	0.69*
	SD	0.03	0.07	0.10	0.03
Urea nitrogen	No.1	18.5	22.4	46.6	28.6
	No.2	18.3	25.2	24.5	21.3
	No.3	19.5	27.5	38.1	23
	Mean	18.8	25.0*	36.4	24.3
	SD	0.7	2.6	11.2	3.8
Creatinine	No.1	0.73	0.68	1.48	1.51
	No.2	0.57	0.47	0.57	0.57
	No.3	0.66	0.69	0.83	0.84
	Mean	0.63	0.60	0.96	0.97
	SD	0.11	0.15	0.47	0.48

*$P<0.05$: Significantly different from pre-value (paired *t*-test).

ketamine (10 mg/kg) decreased systemic blood pressure and systemic peripheral vascular resistivity by about 40% in dogs [16]. Taken together, it is suggested that lower RRI in the ketamine-immobilized condition was resulted from the decreases in systemic blood pressure and heart rate due to higher dose of ketamine, and therefore the dose of anesthesia should be carefully chosen to avoid the effect of anesthesia on RRI when the RRI measurement is conducted under anesthesia condition.

Several factors contributing to variability in RRI including sex and age have already been reported [18]. However, no correlation was noted between RRI and sex or age because small-sized healthy animals with almost animals being 3 years of age were used in this study.

The RRI in the conscious condition in the present study is comparable to that in conscious dogs (0.60–0.62) [20], cats (0.56–0.59) [20], and humans (0.58–0.64) [14]. The cut-off (mean + 2SD) value of RRI indicative of renal disorder [6] was 0.68 in the conscious condition in the present study, comparable to that in dogs (0.73) [21], cats (0.71) [21], and humans (0.70) [17], indicating no apparent difference in the RRI in the conscious state between these species.

A single intravenous injection of cisplatin at 2.5 mg/kg induced renal toxicity in all monkeys, which was characterized histopathologically by minimal to mild renal tubular necrosis and regeneration. In this monkey model of cisplatin-induced acute renal injury, the RRI was increased beyond the cut-off value in the conscious condition (0.68) on Day 7. On the other hand, the conventional biomarkers CRE and UN were slightly increased in 2 out of 3 monkeys with renal injuries. It is well-documented that routine renal function tests based on blood CRE and UN were outdated because they failed to identify early stages of renal dysfunction and structural injuries [1], suggesting that RRI could be more sensitive biomarker for cisplatin-induced renal injury in monkeys. Recently, several urinary biomarkers including Kim-1, albumin, NGAL, and cystatin C have been proposed and approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) [4]. In contrast, kinetics of each biomarker response to various types of renal injuries depends on their mode of action when interpreting the result of the studies. Cisplatin has been reported to accumulate in tubular epithelial cells followed by tubular necrosis, resulting in abnormal permeability of the glomerular basement membrane and eventually decreased renal blood flow [26]. Even though combinational measurements of these biomarkers could supply useful information for detecting potential renal toxicity for new chemical entities, the pathogenesis of cisplatin-induced renal injury could explain the time-course of change in RRI. Taken together, RRI measurement in cynomolgus monkeys is a useful tool for evaluating drug-induced renal injury because of the strong similarities in kidney morphology and hemodynamics to humans [6].

In conclusion, we established a simple and practical method for RRI measurement in conscious cynomolgus monkey without chemical restraint such as sedation or anesthesia. This ultrasonographic method of measuring RRI in monkeys would be a useful tool as an endpoint for evaluating renal injury in conventional toxicological studies.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgments

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