Experimental Animals
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Original

Establishment of a new formula for QT interval correction using a large colony of cynomolgus monkeys

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Abstract: The demand for monkeys for medical research is increasing, because their ionic mechanism of repolarization is similar to that of humans. The QT interval is the distance between the Q wave and T wave, but this interval is affected by heart rate. Therefore, QT correction methods are commonly used in clinical settings. However, an accurate correction formula for the QT interval in cynomolgus monkeys has not been reported. We assessed snapshot electrocardiograms (ECGs) of 353 ketamine-immobilized monkeys, including aged animals, and contrived a new formula for the corrected QT interval (QTc) as a marker of QT interval prolongation in cynomolgus monkeys. Values for QTc were calculated using the formula [QTc] = [QT] / [RR]^n, along with several other formulas commonly used to calculate QTc. We found that the optimal exponent of the QT interval corrected for heart rate, n, was 0.576. The mean value of QTc in healthy monkeys determined using the new formula was 373 ± 31 mm, and there were no significant differences between the sexes. Other ECG parameters were not significantly different between the sexes and there were no age-related effects on QTc. Prolongation of QTc to over 405 ms, as calculated by the new formula, was observed in 50 monkeys with underlying diseases. Additionally, all monkeys with QTc above 440 ms by the new formula had some underlying disease. The results resemble those in humans, suggesting that the new QTc formula could be useful for diagnosis of QT interval prolongation in cynomolgus monkeys.

Key words: cardiovascular disease, cynomolgus monkey, electrocardiogram, QT interval, the corrected QT interval

Introduction

Cardiovascular diseases (CVDs) are a leading cause of human morbidity and mortality. Nonhuman primates are important animals for experimental use, because they have a similar system of systemically metabolic. In CVD studies, animal models involving animals that can be easily bred and can closely simulate human CVDs are necessary. Nonhuman primate models of CVDs can reliably mimic human diseases, so that their causative mechanisms can be investigated and novel drugs, diagnostic procedures and therapies can be developed [17, 29, 39]. As monkeys and humans show similar ionic mechanisms of repolarization, interest in using monkeys for pharmaceutical studies has recently increased [34]. From these, it is important to maintain monkeys that is healthy and usable for research. Also, by detecting diseased monkey early, it can be effectively used for various research.

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For good maintenance and management of experimental animals, it is necessary to detect any underlying diseases or disorders at an early stage in order to increase the efficiency of research.

Experimental models of nonhuman primates are ideal for human medical research, from a viewpoint of anatomy, physiology and genomics. Cynomolgus monkeys (*Macaca Fascicularis*), which were used in this study, are one of the species of nonhuman primates that have been used for the study of various diseases related to aging, such as dementia, because their lifespan is approximately 30 years [33]. Since CVDs are also related to aging, the demand for CVD research using monkey models is also increasing.

Electrocardiograms (ECGs) are very important noninvasive diagnostic examinations for cardiac disease. It is used to diagnose various diseases, such as myocardial infarction, cardiomyopathy and arrhythmias. Among CVDs, arrhythmias account for most cases of sudden death [32, 34]. Hence, research on QT interval prolongation is gaining momentum from various standpoints, such as both its spontaneous and drug-induced occurrence [3, 5, 9, 21, 34, 36]. Many studies are currently conducted based on QT intervals with Holter electrocardiographs [37]. However, ECG monitoring using Holter electrocardiography is very burdensome for monkeys, when the introduction into facility and annual management. On the other hand, evaluation of snapshot ECGs in sedated monkeys is less invasive. However, there are only few reports on monkey ECGs under sedation, and the reports are limited to young monkeys [2, 8, 13, 15, 24–27, 34, 36, 38]. Studies using a wide age range of subjects are required because the incidence of delayed ventricular repolarization increases with aging [28]. Moreover, early diagnosis of any underlying disease is usable to manage and produce of disease models including age-related CVDs. However, one notable issue with using nonhuman primates, such as cynomolgus monkeys, is that their heart rate is very rapid because of their small body size. An increase in heart rate leads to shortening of the RR interval, and, hence, shortening of the QT interval. Therefore, use of a parameter calculated by taking heart rate into consideration when calculating the QT interval, i.e. the corrected QT interval (QTc), is recommended [1, 2, 7, 9, 13, 16, 31, 32, 34, 37]. However, there is no such correction formula for cynomolgus monkeys.

QTc formulas can be classified based on their characteristics as population correction methods, test specific/individual correction methods and Holter bin methods [5, 7, 9]. We examined a population correction method in this study because it is the most popular method and can be used at any time.

Here, we aimed to determine a highly sensitive method to detect CVD in cynomolgus monkeys using snapshot ECGs, focusing on the QT interval, when the introduce of animals to facility in the present study. This report used over 300 monkeys ranging in age from young (1.2 years old) to senior (36.4 years old) monkeys, and investigated the utility of electrocardiographic reference values and formulas for calculating QTc as a marker of arrhythmia in cynomolgus monkeys. This is the first report to investigate the best QTc calculation formula using standard ECGs of cynomolgus monkeys of a wide age range. Due to the convenience and simplicity of ECG monitoring, ECGs are commonly used to identify monkeys with various underlying diseases, facilitating management of the monkey colony. This is likely to be useful because the demand for monkey data for advanced medical sciences and pharmaceutical studies has been recently increasing and the data of this report will significantly contribute to achieving this goal.

Materials and Methods

Animals

The ECG data of 353 monkeys (191 females, 162 males), weighing approximately 1.2 to 10 kg and ranging in age from 1.2 to 36.4 years, were examined in this study (Table 1). The monkeys were housed and bred at the breeding colony of Tsukuba Primate Research Center. All monkeys were individually housed in stainless-steel cages under the following conditions: temperature, 23–27°C; humidity, 50–70%; 12 air changes/h; and 12/12-h light/dark cycles, and were fed 70 g of commercial monkey chow (CMK-2; CLEA Japan, Inc., Tokyo, Japan) and 200 g of fruit daily, unless otherwise indicated. All the monkeys were evaluated by regular annual examinations, including chest auscultation and palpation to assess their physical condition. After the ECG was monitored, monkeys clinically diagnosed with cardiovascular dysfunction underwent other examinations, such as common blood tests, echocardiography,

Table 1. Characteristics of the cynomolgus monkeys used in this study

	Animals	Female 191	Male 162	Total 353
Weight (kg)	Mean ± SD	3.8 ± 1.2	4.7 ± 1.6	4.2 ± 1.5
	Range	1.2–8.1	1.4–10.0	1.2–10.0
Age (years)	Mean ± SD	16.1 ± 9.0	9.7 ± 8.0	13.1 ± 9.2
	Range	1.2–36.4	1.3–33.1	1.2–36.4

The study included a large number of monkeys and covered a wide age range.

chest X-ray and blood pressure measurements. This study proceeded according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center, the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [10, 11] and the Guide for the Care and Use of Laboratory Animals [12]. The Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation, Health and Nutrition (NIBIOHN, Osaka, Japan) approved the study protocol.

ECG values

ECGs were obtained in the supine position under immobilization in this study [34]. Each monkey was immobilized using 10 mg/kg of ketamine hydrochloride intramuscularly (Ketalar; Daiichi Sankyo Propharma, Tokyo, Japan). Standard 6-lead ECGs were recorded using a 3-channel electrocardiograph (D300; Fukuda ME, Tokyo, Japan). The leads were set at the commonly used positions, i.e. the right armpit, left armpit, right groin (ground) and left groin.

For each animal, five QT and RR intervals were manually measured, and their values were averaged to obtain a single value. Additionally, the other parameters were measured automatically by the device. Three waveforms were averaged by the device, and the averaged value including T wave which all unclear waves were removed was used as a parameter.

QT interval correction methods

The QT interval is usually measured automatically by an electrocardiograph. QTc is affected by heart rate and the RR interval. However, ECG device that are usually used in humans, cannot measure QT and RR interval of cynomolgus monkey accurately. In this study, we measured each ECG value and calculated QTc using several formulas commonly used in human medicine [3, 6, 23]. First, the QT interval and heart rate were measured from the ECG. Second, we applied the results of these measurements to various previously reported correction formulas for humans, and examined the calculated values for QTc. The correction formulas that we used in this study included Bazett's formula $[QTc] = [QT] / [RR]^{1/2}$, Fridericia's formula $[QTc] = [QT] / [RR]^{1/3}$, Framingham's formula $[QTc] = [QT] + (1 - [RR] \times 0.154)$, Hodges formula $[QTc] = [QT] + 1.75 \times ([\text{heart rate}] - 60)$, and Yoshinaga's formula $[QTc] = [QT] / [RR]^{0.31}$ [23].

Next, we created a new formula that we modified from other formulas, namely Bazett's, Fridericia's and Yoshinaga's formulas, and examined QTc using this new formula.

Finally, the reference value of QTc using all of the methods was determined based on its frequency distribution.

Statistical analysis

Bilateral Welch's *t*-test was performed on each QTc value to assess differences between sexes. *P* values of 0.05 or less were considered statistically significant. The correlation between RR interval and QTc in each formula was assessed using Pearson's correlation coefficient analysis. Pearson's correlation coefficient was also used to assess the correlations of other ECG parameters with age and weight. Graphical data were obtained using calculation software (Microsoft Office Excel 2016; Microsoft Corp., Redmond, WA, USA). All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (ver. 3.3.2, The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander (ver. 2.3-0) designed to add statistical functions frequently used in biostatistics [20].

Results

We evaluated Leads I to III of the ECG in this study. We found no sex-related differences in ECG parameters. Figure 1 shows examples of standard and abnormal ECGs from cynomolgus monkeys that were assessed in this study. The standard ECG from a cynomolgus monkey is similar in shape to the human ECG (Fig. 1A), while the abnormal ECG demonstrates QT prolongation and low-voltage of the R wave (Fig. 1B). The QRS wave of cynomolgus monkeys is known to have a low amplitude, as was also seen in the healthy monkeys in this study [18, 35]. Although each interval was shorter than that in humans, the amplitudes were similar to those in humans. Most monkeys showed the highest amplitude in Lead II, and the heart's electrical axis was similar to that in humans.

Most parameters, especially the R wave, of normal monkeys were shown by Holter ECG to be of somewhat lower amplitude compared with the human ECG. Further, snapshot ECGs under ketamine immobilization resembled the Holter ECG of cynomolgus monkeys (Supplementary Fig. 1). Although ketamine is known to increase heart rate, previous studies reported that ketamine immobilization does not affect ECG parameters [18, 35], suggesting that evaluation of snapshot ECG parameters under ketamine immobilization is useful for detecting underlying diseases (Supplementary Table 1).

Bazett's formula has long been used for various ECG evaluations. However, previous reports only included

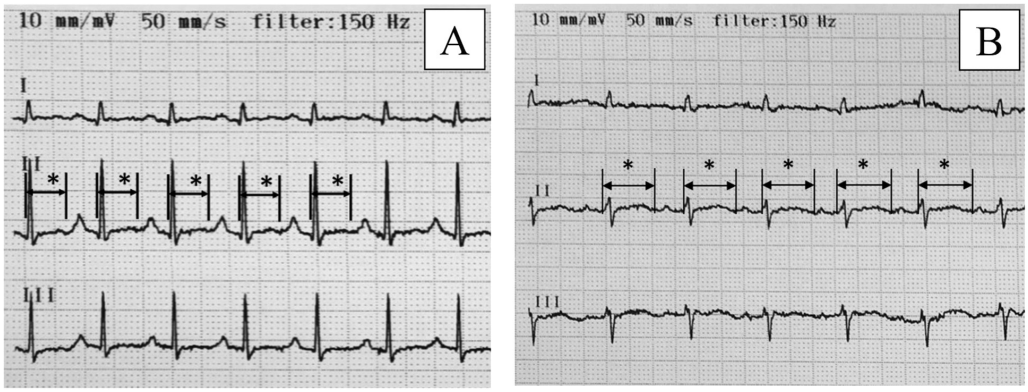

Fig. 1. Comparison between abnormal and normal electrocardiograms (ECGs) in cynomolgus monkeys. The abnormal ECG demonstrates clear QT interval prolongation as compared to the normal ECG. The normal ECG from a healthy cynomolgus monkey is similar to a human ECG, although the heart rate is faster (A). This abnormal ECG shows remarkable QT prolongation, prolongation of the QRS interval and left axis deviation (B). This monkey was diagnosed with severe dilated cardiomyopathy.

young animals for toxicological examinations. Therefore, there are no reports including older animals, as in our study. In this study, we tried to establish a QT correction formula for a wide age-range of animals, including older animals.

Comparison of QTc calculated using the six formulas showed that Bazett's formula was least influenced by the RR interval. We then modified Bazett's formula to derive a formula that was even less affected by the RR interval. Bazett's formula is $[QTc] = [QT] / [RR]^n$, in which the value of the exponent, n, is 1/2. Since we considered that a more suitable formula could be derived by finding an optimal value for n, we assessed a formula using the correlation coefficient between the QT interval and RR interval as the exponent 'n'. We found the value of 'n' to be "0.576". Plots of correction results using several formulas, including the new one, are shown in Fig. 2. As seen in the graph, the new formula can stably correct QT interval even in older monkeys.

When this formula was used to calculate QTc, there were no sex differences in ECG parameters (Table 2, Fig. 3A). Further, there were no differences in QTc in relation to age (Fig. 3B). Similarly, there were no body weight-dependent effects on QT interval (Fig. 3C). The QTc using the new formula was 373 ± 31 ms (for both sexes). The data derived using the new formula and the plotted distribution were similar to those previously reported for humans (Fig. 3A) [19]. Additionally, we found that many monkeys with underlying diseases, such as dilated cardiomyopathy, diabetes mellitus and valvular heart disease, demonstrated prolonged QTc intervals of more than 405 ms (Fig. 3D). After ECG evaluation, pathological analysis of the heart was performed in all animals with a QTc of over 405 ms by our new formula, to evaluate the presence of underlying diseases. The results of

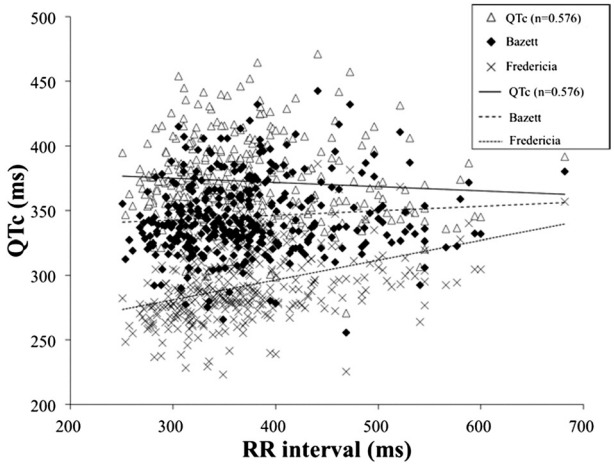

Fig. 2. Comparison between the corrected QT interval (QTc) calculated using the new formula and those calculated using other formulas. QTc calculated using our new formula exhibited ideal correction as compared to QTc calculated using other formulas. Only three formulas, including ours, showed especially good correction. Bazett's formula and the new formula had almost no correlation with the RR interval, which is ideal. This indicates that these two are the most suitable formulas for calculating QTc.

pathological analysis revealed that the most common underlying disease in cases with QTc over 405 ms was dilated cardiomyopathy, and diabetes animals were classified to type 2 diabetes mellitus (Figs. 3D and 4).

Discussion

Standard ECG values in cynomolgus monkeys

Although the ECGs of cynomolgus monkeys have been evaluated before, previous studies involved fewer subjects and only young monkeys [1, 2, 7, 9, 34]. This study is the first large scale ECG study in cynomolgus monkeys of a wide age range. This is also the first study

Table 2. Electrocardiogram (ECG) parameters and the corrected QT interval (QTc) calculated using six formulas in 353 cynomolgus monkeys

	Female	Male	Total	Correlation with RR interval	Correlation with age	*P*-value
$QTc^{0.576}$	371 ± 31	375 ± 32	373 ± 31	–0.07^{+}	0.01	
QTcB	342 ± 29	348 ± 29	345 ± 29	0.09^{+}	0	
QTcF	288 ± 26	297 ± 27	292 ± 27	0.41	–0.02	**
QTcFr	310 ± 21	306 ± 19	303 ± 21	0.48	–0.03	***
QTcH	176 ± 51	188 ± 45	197 ± 45	–0.97	0.05	***
QTcY	290 ± 27	285 ± 26	281 ± 27	0.44	–0.03	**
Heart rate (beats/min)	173 ± 26	161 ± 31	167 ± 29	–0.97	0.05	***
RR (ms)	356 ± 61	389 ± 79	371 ± 72	1	–0.07	***
QT (ms)	204 ± 25	216 ± 29	210 ± 28	0.76	–0.05	***

The new formula and Bazett's formula could appropriately correct the QT interval. +: No correlation. **: $P<0.01$, ***: $P<0.001$. $QTc^{0.576}$: QTc calculated using the new formula (exponent=0.576). QTcB: QTc calculated using Bazett's formula. QTcF: QTc calculated using Fridericia's formula. QTcFr: QTc calculated using Framingham's formula. QTcH: QTc calculated using Hodges' formula. QTcY: QTc calculated using Yoshinaga's formula. QT: manually measured QT interval.

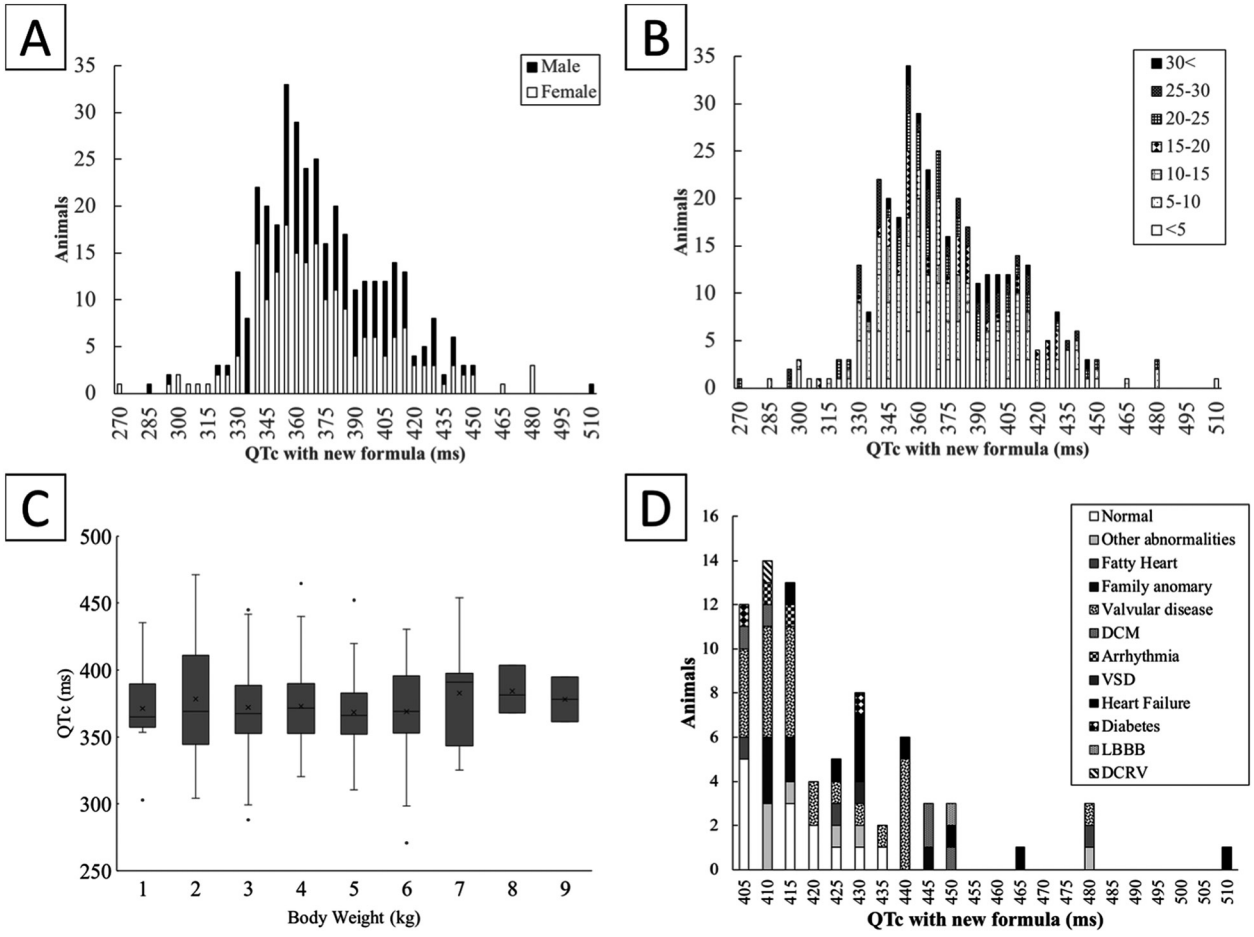

Fig. 3. Correlation between the corrected QT interval (QTc) calculated using the new formula and underlying diseases, sex, body weight and age. QTc calculated using the new formula was useful in the diagnosis of underlying diseases. This figure shows the distribution of QTc in relation to sex in all the samples; there were no sex-related differences in QTc in this study (A). Body weight and age-dependent effects were mostly absent (B, C). Relationship between monkey QTc intervals longer than 405 ms and underlying diseases (D). Electrocardiograms (ECGs) with QTc intervals longer than 405 ms were observed in monkeys with various cardiovascular and systemic diseases, indicating that the new correction formula can correct QT interval appropriately. LBBB: Left bundle branch block, VSD: Ventricular septal defect, DCM: Dilated cardiomyopathy, DCRV: Double chambered right ventricle.

to determine reference ECG values in immobilized cynomolgus monkeys for future use in pharmacological, physiological and other studies.

The cynomolgus monkeys in the present study were immobilized with ketamine hydrochloride prior to measuring ECGs. Use of ketamine for immobilization is safe

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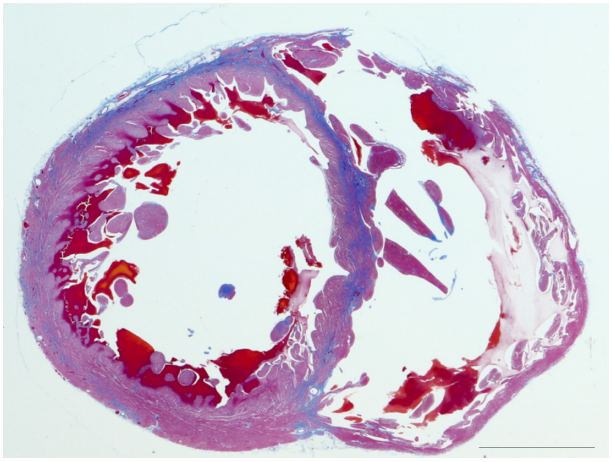

Fig. 4. Microscopic evaluation of cardiac tissue in animals who presented with the corrected QT interval (QTc) of over 405 ms using the new QT correction formula. Several monkeys who showed QTc of over 405 ms using the new formula demonstrated dilated cardiomyopathy on microscopic evaluation. This microscopy obtained the monkey that showed QT interval prolongation. Dilated cardiomyopathy was diagnosed on the basis of observation of fibrosis extending over both ventricles and thinning of the ventricular wall (Bar=10 mm). Since these findings were found in the group with QTc of over 405 ms using the new formula, it suggests the utility of this formula.

and does not affect the ECG or heart rate in nonhuman primates [18, 35]. Assessment of the ECG under anesthesia is a common and basic inspection method in nonhuman primates [2, 34]. Detection of any underlying disease at an early stage under reproducible, safe and non-stressful conditions will facilitate management of the animals and enable creation of various experimental models of cynomolgus monkeys [35].

Here, we evaluated the correlation between various ECG parameters and age and weight. Our study showed no significant correlation between ECGs and age or weight (Figs. 3B and C). In humans, the PQ interval and duration of P are known to be prolonged with aging [3, 6, 23]. Our results, on the other hand, suggest that aging is not associated with a prolonged PQ interval and duration of P in cynomolgus monkeys. Prolongation of the PQ interval and P duration with aging in other macaques has also never been reported. Hence, it was necessary to investigate the PQ interval and duration of P with aging. The mean amplitude of the R wave in this study was higher than in previous studies, while the amplitudes of other waves in the ECG were similar to those previously reported. [1, 2, 7, 8, 34]. However, the results of previous studies were affected by sample size. The present study used over 300 monkeys, all of whom underwent periodic health examinations, and none of whom had irregular cardiovascular symptoms or systemic abnormalities. This increases the reliability of our data.

The heart rate of the cynomolgus monkeys in the present study (females: 173 ± 26 bpm, males: 161 ± 31 bpm) was slower than previously reported, although the other parameters, such as amplitude of P wave and T wave, were almost the same as those in previous reports [1, 2, 7, 8, 34]. In this study, we evaluated cynomolgus monkeys in the supine position. Heart rate is known to be affected by position, becoming slower in the supine position. Hence, the slower heart rate in this study compared to a previous study was likely because the ECGs were recorded with the monkeys in the supine position [34].

New QTc formula for cynomolgus monkeys

The amplitudes and durations of the various components of the ECG are useful for detecting cardiac diseases, such as cardiomyopathy. In humans, measurement of the QT interval of the ECG is considered especially important in the diagnosis of many cardiac diseases [22]. Researchers commonly use QTc rather than QT in their studies, because the QT interval is affected by heart rate. Bazett's formula ($[QTc] = [QT] / [RR]^{1/2}$) and Fridericia's formula ($[QTc] = [QT] / [RR]^{1/3}$) are the common formulas used for calculating QTc. Bazett's formula is used in adults, while Fridericia's formula is used in children and in patients whose heart rate tends to fluctuate. Yoshinaga's, Hodges' and Framingham's formulas are also used for QT correction. Yoshinaga's formula uses a different exponent from Bazett's and Fridericia's formulas; however, it has not been used much in drug discovery research because it overestimates QTc when the heart rate is rapid. Bazett's formula is commonly used to correct QT interval, although it is most often used in toxicological examinations and no previous reports have included older monkeys, unlike in this study. Hodge's and Framingham's formulas are correction methods that apparently do not overestimate QTc at rapid heart rates as compared to the formulas using an exponential function [23]. However, these formulas were not able to accurately correct the QT interval and seemed to correlate with the RR interval in our study. Our results suggested that Bazett's formula (r=0.087) is the most appropriate from among the existing formulas for calculating QTc (Fig. 2), since it does not correlate with the RR interval.

Additionally, we used the correlation between RR and QT intervals to obtain the exponent for a more ideal QT correction formula in this study, and arrived at a value of 0.576. Substitution of this new exponent into Bazett's formula, $[QTc] = [QT] / [RR]^{n}$, resulted in a more accurate correction of the QT interval (r=−0.075). In this new formula, the QT interval is less affected by heart rate, making it superior to Bazett's formula.

In the present study, we also investigated sex-related differences in QTc. Figure 3A shows sex-related QTc distribution, which showed no sex differences in QTc in this study. Additionally, no significant age- and weight-related difference in QTc was detected in this study (Figs. 3B and C). As shown in Fig. 3B, we also investigated the relationship between QTc of over 405 ms and several disease conditions. After the ECG analysis, we reconducted medical examination and cardiac-focused examination, such as chest X-ray, cardio-specific blood tests and echocardiography. As shown in the graph, many of the monkeys had cardiovascular and other systemic diseases, such as ventricular septal defect, dilated cardiomyopathy, heart failure and diabetes mellitus. Additionally, pathological analysis of monkeys in whom QTc was over 405 ms revealed that the most common abnormalities were dilated cardiomyopathy and Type 2 diabetes mellitus (Fig. 4). Moreover, mitral stenosis was the most commonly diagnosed valvular disease due to progression of age-dependent fibrosis in cardiac valves. Further, all monkeys with QTC over 440 ms had underlying diseases. Since not only structural anomalies of the heart, but also electrical abnormalities such as arrhythmia and systemic diseases such as diabetes were widely included, using QTc of 405 ms or more as a diagnostic criterion will be useful for early detection of various diseases. These results are similar to those reported in human research, suggesting that the data of this study could be useful in medical research and as part of routine medical care of nonhuman primates used in research [3, 6, 23]. Furthermore, this report could be used to detect several diseases for various researches.

In this study, we compared ECG parameters, including mean QTc calculated using the different formulas (Table 2). The QTc in this study was longer than those previously reported, because both the QT interval and RR interval were measured manually in this study. Manual measurements allow more accurate assessment of QT and RR intervals [30]. Furthermore, the QTc obtained using our formula was similar to those in adult humans [3, 23]. The QTc values in previous studies were approximately 200–300 ms when calculated using Bazett's and/or Fridericia's formulas. Those calculated by the new formula in this study were approximately 350–400 ms. This result indicates that our formula provides stable correction of the QT interval. This value of QTc is closer to that in humans [3, 6, 23], suggesting that the new formula is appropriate for clinical use and usable in longevity research and advanced medical science.

Previous studies have described significant sex and age-related differences in the QT interval in humans. However, graphical evaluation of the relationship between QT interval and age shown in Fig. 3B revealed no age-dependent effect on QTc calculated by our formula in this study. A study on prolonged QTc syndrome in humans found that the disease-related genes are frequently transmitted in women [4, 19]. Although there are a few reports that the genes are paternally imprinted during early ontogenesis in human genome research, the reports are not conclusive, and the effect on actual pathophysiological phenotype is still unknown [14]. In monkeys kept in laboratory facilities, their amount of activity is smaller and they are more relaxed and in a more constant living environment than wild animals. That is suggested as one cause for the difference between humans and monkeys. Although human and nonhuman primates have very few differences compared to between humans and mice and rats, it is also possible that genes related to the QT interval have different inheritance patterns. Genome research in nonhuman primates is currently still in its elementary stages, and it is expected that sex differences in QTc will be clarified in the future.

In conclusion, we established a new formula to detect QT prolongation syndrome in cynomolgus monkeys, and determined standard ECG values in cynomolgus monkeys. These data will be useful for clinical and pharmaceutical research, and might be an important tool for the management of experimental nonhuman primates.

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