Studies on cardiovascular and autonomic nervous effects of trichothecene mycotoxin in the rat

（トリコテセンマイコトキシンのラット心臓血管系および自律神経系に及ぼす影響に関する研究）

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Contents

Abbreviation

AF	=	aflatoxins
ANOVA	=	analysis of variance
ANS	=	autonomic nervous system
A-V block	=	second-degree atrioventricular block
BP	=	blood pressure
CO	=	cardiac output
DMEM	=	dulbecco’s modified eagle medium
DMSO	=	dimethyl sulfoxide
DON	=	deoxynivalenol
ECG	=	electrocardiogram
EDV	=	end diastolic velocity
EF	=	ejection fraction
ETS	=	electron transport chain
F	=	fumonisins
FCCP	=	carbonyl cyanide-*p*-trifluoromethoxyohenylhydrazone
FFT	=	fast Fourier transform
FS	=	fractional shortening
HF	=	high frequency
HR	=	heart rate
HRV	=	heart rate variability
IM	=	inner membrane
IP	=	intraperitoneal injection
IV	=	intravascular injection
LF	=	low frequency
MBP	=	mean arterial blood pressure
MV	=	mean velocity
OCR	=	oxygen consumption rate
OT	=	ochratoxins
PBS	=	phosphate buffered saline
PI	=	pulsatility index
PSV	=	peak systolic velocity
PVC	=	premature ventricular contraction
RC	=	reserve capacity

RI	=	resistive index
ROS	=	reactive oxygen species
S.C.	=	subcutaneous injection
SV	=	stroke volume
SVE	=	supraventricular extrasystole
VE	=	ventricular extrasystole and ventricular tachycardia
XF	=	extracellular flux
ZEN	=	zearalenone

Abstract

This study aimed to elucidate toxicities and the toxic mechanisms of representative trichothecene mycotoxins, i.e., T-2 toxin and deoxynivalenol (DON), to the cardiovascular and autonomic nervous system. In order to achieve this goal, the scheme was considered several experiments consisted of both *in vivo* and *in vitro* methods. *In vivo* studies a series of experiments were conducted in Wistar strain rats as experimental animals under the anesthetized or conscious condition, while *in vitro* study, the cardiomyocyte was obtained from neonatal SD strain rats. The cell culture assay was carried out to identify a direct toxicity to the rat cardiomyocyte due to the trichothecene mycotoxins.

In *in vivo* study, changes in the electrocardiographic (ECG) parameters and the autonomic nervous activity that were affected by administration of trichothecene mycotoxin were mainly investigated by a telemetry method. In these experiments, possible contributions of baroreceptor reflex that result in changes of heart rate (HR) by responding to blood pressure (BP) changes as well as concurrent alterations in the autonomic nervous activity measuring from the heart rate variability (HRV) were examined. Moreover, the echocardiography was employed to investigate circulatory changes including cardiac functions and systemic vascular resistance after the toxin administration. In *in vitro* study, changes in mitochondrial respiratory function, which were measured as oxygen consumption rate (OCR), in cultured cardiomyocytes were investigated after direct application of T-2 toxin and DON to the cardiomyocytes.

First, acute cardiovascular changes in response to subcutaneous administration of T-2 toxin (0.5 and 1.0 mg/kg) and DON (0.5, 1.0 and 2.0 mg/kg) were observed for 3 hours in anesthetized rats. T-2 toxin induced an increase in HR that begun at 60 min

while especially QRS duration and QT interval in the ECG wave-components tended to increase at least 30 min after the administration. Moreover, the occurrence of arrhythmia was recognized within 3 hours of observation in the administration of T-2 toxin. As regards DON administration, a decrease of HR starting from 90 min with a prolongation of PR interval and QRS duration, being accompanied by the occurrence of arrhythmia, was observed. Furthermore, these two toxins induced a similar change of blood pressure characterized by an increase in the systolic, diastolic and mean arterial blood pressure after 30 min of the toxin administration. From these acute experiments, it was found that T-2 toxin and DON possess the toxicity to produce the functional alteration in the cardiovascular system.

On the basis of the results described above, in the next step the experiments with long-term observation for toxicities by the two mycotoxins were conducted in order to clarify reversible or irreversible alterations of the cardiovascular functions in unanesthetized and unrestrained rats. In this study, telemetric measurements of ECG and the analysis of heart rate variability (HRV) were performed to record long-term responses to T-2 toxin and DON in freely moving rats with subcutaneous administrations of toxins. Subcutaneous injections of T-2 toxin (0.1 and 0.5 mg/kg) and DON (0.5, 1.0 and 2.0 mg/kg) were done twice at an interval of 3 days. A marked change in HR was observed in especially T-2 toxin administration, where T-2 toxin and DON both induced the increase in HR after 90 min following the administration. In this long-term observation, the cardiovascular toxicity due to T-2 toxin was found to be lasted for as long as 3 days, being identified by the increase in HR, significant decrease in HRV components and occurrences of various types of arrhythmia within 3 days of observation at dose of 0.5 mg/kg. The change in HR and HRV components by DON

administration lasted for approximately 12 hours which was shorter than that in T-2 toxin. Moreover, DON-induced abnormalities, i.e., changes in HRV components, arrhythmias, seemed to be reversible changes since all the ECG parameters including HR could recover to its normal values within 3 days of observation. From the results of these studies in this section, it was elucidated that T-2 toxin has a potent and long-lasting toxicity which is represented by significant changes in HR, autonomic nervous activity and frequent occurrence of arrhythmia and also elucidated that such changes in T-2 toxin is significantly greater than those produced by DON.

As for the mechanism of HR changes and the occurrence of arrhythmia induced by T-2 toxin and DON in the present study, the participation of the autonomic nervous activity and/or direct action of those toxins on the heart were assumed as causal factors of such cardiac alterations. Therefore, in the next step, the effect of autonomic nervous blockades on the HR change and occurrence of arrhythmia as well as changes of HRV was examined in rats in which the osmotic mini-pump containing atropine as a parasympathetic nerve blockade or propranolol as a sympathetic nerve blockade was implanted and T-2 toxin (0.5 mg/kg, s.c.) or DON (2.0 mg/kg, s.c.) was injected. As a result, the increase of HR due to T-2 toxin and DON administration were inhibited by the continuous administration of propranolol (100 mg/kg/day). Moreover, the arrhythmias, especially second-degree AV block and sinus bradycardia, which responded to T-2 toxin were significantly reduced by both the blockades. On the other hand, the occurrence of ventricular extrasystole was significantly increased by the administration of atropine (20 mg/kg/day). In addition, the LF power and LF/HF ratio were reduced by the atropine administration in both toxins, while the HF power was also reduced by atropine in DON. According to these data, it was clarified that in

unanesthetized rats T-2 toxin and DON produced the HR change, arrhythmia with a conduction disturbance and changes in HRV via the alteration of the autonomic nervous activity and some cardiac abnormalities such as ventricular extrasystoles may be provoked by not only the autonomic nervous system but also a possible direct action of toxins to the cardiac tissue.

In *in vivo* study, the dynamic circulatory changes including cardiac contractility, blood flow velocity, cardiac output and peripheral vascular resistance were also examined in rats with administration of T-2 toxin (0.1 mg/kg, 0.5 mg/kg, s.c.) with the aid of echocardiogram equipped with B-mode, M-mode and pulse Doppler analysis. This echocardiographic observation revealed a tendency for increase in cardiac output and a significant increase in mean blood flow velocity in the common carotid artery and femoral artery in rats with administration of T-2 toxin at 48 h after the injection, accompanying by a tendency for increase in HR and fractional shortening and an increase or decrease in resistance index in the common carotid artery and femoral artery at the dose of 0.5 mg/kg. After the echocardiogram examination the blood serum sample was collected for measurement of oxidative stress. This measurement resulted in a significant increase of amount of reactive oxygen species (ROS) without a change of antioxidative parameter in rats administered with T-2 toxin. From these experiments, it was clarified that the dynamic change in the circulatory system was produced by T-2 toxin, accompanying by the concurrent increase in the oxidative stress in the blood.

On the basis of the results described above and from the past reports, I assumed that these trichothecene mycotoxins may have a property of cellular toxicity through the direct action of the toxins to the cardiac muscle. The cultured primary cardiomyocyte isolated from newborn rats were assigned for *in vitro* experiments on the toxicity of T-2

toxin and DON to the mitochondria respiratory function in which the oxygen consumption rate (OCR) was measured at 24 h after the onset of T-2 toxin application. As a result, a significant decrease in baseline OCR was recognized at a concentration of 6×10^{-4} μM of T-2 toxin and at 0.78 μM of DON. Furtheremore, the ATP-linked OCR in response to oligomycin was significantly decreased at 6×10^{-5} μM of T-2 toxin and at 0.78 μM of DON, while the similar OCR change was observed for the response to FCCP that indicated the reserve capacity of electron transport system in the mitochondria.

In conclusion, the present study demonstrated that T-2 toxin and DON have considerable toxic properties for the cardiovascular system involving the appearance of potent arrhythmia, marked alterations in the autonomic nervous function, and cardiac cellular toxicity and that the extent of toxicity is much greater in T-2 toxin than that in DON.

CHAPTER 1

GENERAL INTRODUCTION

Mycotoxin is a toxic secondary metabolite produced by fungi species. Approximately 25% of the world`s food crops are contaminated by fungi that produce toxin (Rotter et al., 1996). Examples of mycotoxins of greatest public health and agroeconomic significance include aflatoxins (AF), ocharatoxin (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins and ergot alkaloids (Hussein and Brasel, 2001). Negative effects of mycotoxins on humans focus on a possibility to cause cancer. The World Health Organization International Agency for Research on Cancer (1993) evaluated the carcinogenic potential of AF, OT, trichothecenes, ZEN, and F. Naturally occurring AF were classified as carcinogenic to humans (Group 1) while OT and F were classified as possible carcinogens (Group 2B). Trichothecenes and ZEN, however, were not classified as human carconigens (Group 3). While in animal, variable responses have been shown with all mycotoxins. For example, pigs have been shown to be very sensitive to T-2 toxin, deoxynivalenol (DON) (Friend et al., 1992), and ZEN (Biehl et al., 1993). Pigs that were fed with T-2 and DON contaminated-feed showed lower final body weight with the lesions on the mucosa of the pars esophageal region (Friend et al., 1992). Poultry are also adversely affected by both T-2 toxin and DON but are very resistive to the estrogenic effects of ZEN (Cheeke, 1998). Various degrees of mycotoxicoses from natural sources occur in different animal species because of the wide range of feed ingredients used and the differences among and within species.

Considering its frequency of occurrence and average concentration, T-2 toxin and DON or ‘vomitoxin’ appear to be one of the most pathogenic and important mycotoxin present in cereal commodities (Gareis et al., 2003). These 2 types of mycotoxin are produced by *Fusarium* fungi or known colloquially as ‘red fungi’ and belong to a tricothecene mycotoxin group. T-2 toxin (Figure 1.1) is classified into

tricothecene group type A, while DON (Figure 1.2) is a member of tricothecene group type B. T-2 toxin (MW=466.6) is the most toxic and is soluble in non-polar solvents (e.g. ethyl acetate and diethyl ether), whereas DON (MW=296.3) is soluble in polar solvents such as alcohols (Trenholm et al., 1986). This type of mycotoxin is produced by a variety of different fungi such as *Fusarium*, *Trichoderma*, *Myrothecium*, *Verticimonnosporium*, and *Stachynotrys* (Ballantyne et al., 2009). These toxins mainly cause symptoms in a digestive tract. Especially, T-2 toxin has been reported as a life-threating mycotoxin since it causes severe illness in humans and animals by causing the entire digestive damage following with rapid death due to internal hemorrhage (Borison et al., 1991). Functional and morphological changes of the cardiovascular system have also been reported as effects of T-2 toxin. The cardiovascular changes, if they are potent, are noteworthy since such effects have not so frequently been described as health effects of other mycotoxins and arrhythmias such as ventricular tachycardia, if they occur, can be a lethal factor in both humans and animals. The earlier studies on the cardiovascular system in experimental animals revealed a significant decrease in systemic blood pressure and systolic left ventricular pressure in anesthetized rats at 24 hours following subcutaneous injection of 1 or 2 mg/kg of T-2 toxin (Magnuson et al., 1987) and also an increase in systemic blood pressure, arrhythmia lasting for 6 to 8 hours and thereafter hypotension in rats administered T-2 toxin at doses ranging from 0.5 to 2 mg/kg (Feuerstein et al., 1985). Reflex effects of the autonomic nervous system on the cardiac conduction system and some inhibitory effects on action potential in the dog have been reported (Bubien & Woods, 1986, 1987). In addition, it was shown that cultured myocytes ceased beating at 10 to 30 min after T-2 toxin application at concentrations of 250 μg/ml or higher (Yarom et al., 1986).

A review of the literature regarding trichothecene mycotoxin toxicity indicates that many researches have been focused on short-term response following toxin administration. This is feasible from its rapid and efficient absorption ability. Comparison between T-2 toxin and DON, T-2 toxin is more rapidly absorbed than DON after its ingestion by most species, its plasmatic half-life is less than 20 min (Beasley et al., 1986, Larsen et al., 2004). The distribution in organs of trichothecene mycotoxin has been believed that it is very rapidly and dramatically eliminated without accumulation in organism (Eriksen and pettersson, 2004). A very low concentration of DON and its derivatives are already found in plasma 8 h after DON ingestion; 88-100% of the plasmatic concentration has disappeared after 24, to almost zero at 72 h (Azcona-Olivera et al., 1995, Eriksen et al., 2003, Danicke et al., 2004). In my previous experiments the blood concentration of DON in rats was shown as a maximum at 30 minutes and decreased to 14 to 23 % of the maximum at 180 minutes after the subcutaneous administration of DON with a concentration of 0.1 to 2.0 mg/kg.

Even if DON and its metabolites are no longer detected in plasma after 8 h, their presence in urine 48 h after ingestion demonstrates storage in tissue and slow rejection and/or binding to blood proteins (Eriksen et al., 2003). While at a low dose (0.15 mg/kg BW/day) of T-2 toxin more than 95% of the ingested T-2 toxin is eliminated within 72 h, the increased dose up to 0.6 mg/kg BW the toxin is more slowly eliminated, being probably due to the metabolism saturation (JECFA, 2002). On the basis of these reviews, it is reasonable that the observation time for cardiac function in present study was setting to 72 h and defined as long-term response.

Many physiological parameters on the cardiovascular organ have been employed for assessments of various environmental substances and drugs on

experimental animals and humans. However, the mechanism of cardiovascular toxicity due to T-2 toxin and DON has not been well clarified. For instance, the relationship of the autonomic nervous activity and HR changes with or without an occurrence of arrhythmias has not been explored. In a case of the occurrence of arrhythmia, the pathophysiological mechanism should be identified because of its significance on health hazard. As regards such toxicity, I also consider the mechanism for direct action on the heart by these toxins. In a previous report Yarom et al. (1983) studied the effects of T-2 toxin on the isolated perfused heart in rats. In this literature review, Langendorff technique was performed with an aim to measure the heart contractility with simultaneous observation of electrocardiogram (ECG). In addition, the heart tissues were collected and the sections were made to study on histological changes in the hearts. It was recognized from these studies that the contractility of the heart was decreased by perfusion of T-2 toxin, being accompanied by concurrent changes in ECG, and the myocyte swelling was generated. According to the above reports, it is considerable that T-2 toxin may have a direct toxic effect on the myocardium as well as functional alterations in the autonomic nervous regulation of the heart.

As for the toxicity to the other cells, it has been described that the trichothecene mycotoxin induces cellular changes in various cell types like HL60, Jurkat, U397, Vero cells and human hepatoma cells (Baltriukiene et al., 2007, Bouaziz et al., 2008, Ma et al., 2012). Most of these studies have focused on apoptotic pathways that affected by the toxin. Besides these findings, it has been also known that both DON and T-2 toxins possess a characteristic as a protein and nucleic acid synthesis inhibitor (Ueno et al., 1973, Shifrin and Anderson, 1999, Doi et al., 2006, Paterson and Lima, 2010).

The main objectives of the present thesis were to investigate cardiac and autonomic nervous responses against the exposure to 2 representative mycotoxins in the trichothecene group and attempt to elucidate the mechanism of the possible toxicity caused by acute and long-term exposures to the toxins through *in vivo* and *in vitro* experiments.

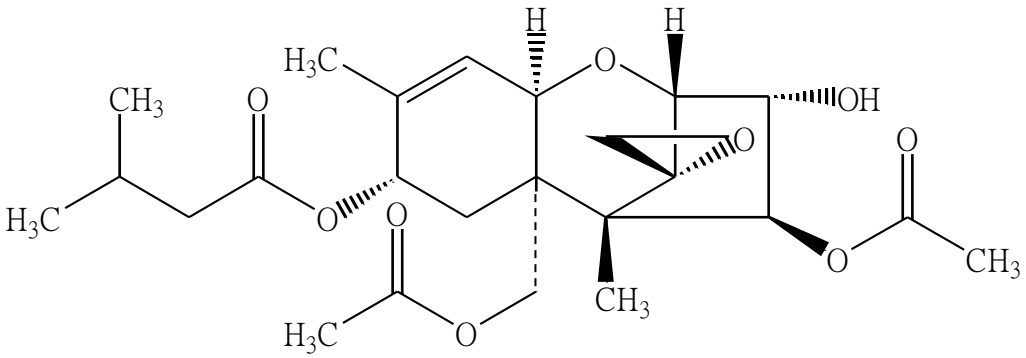


Figure 1.1 Chemical structure of T-2 toxin

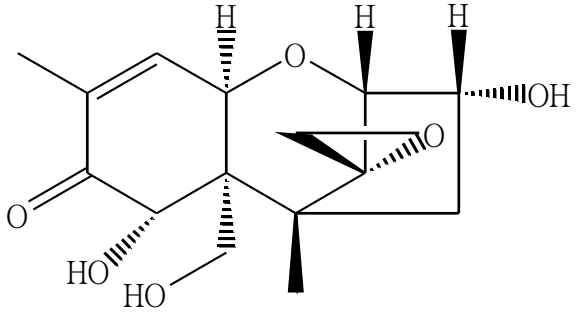


Figure 1.2 Chemical structure of deoxynivalenol (DON)

CHAPTER 2

ACUTE RESPONSES OF HEART RATE, BLOOD PRESSURE AND ECG WAVEFORM FOLLOWING THRICHOTHECENE MYCOTOXIN ADMINISTRATION

2.1 Introduction

Studies in this chapter aimed to evaluate acute effects of the trichothecene group of mycotoxins, T-2 toxin and DON, on cardiovascular functions in anesthetized rats.

T-2 toxin has been suggested to possess potent cardiovascular toxicities that may be not ignored as mentioned below. Wilson et al. (1982) reported that rat that received 2 mg/kg intragastric dose of T-2 toxin caused hypertension and cardiovascular lesions. An occasional contamination of food with *Fusarium spp*. might be the cause of similar lesions also in human (Schoental, 1980, 1981). Acute parenteral administration of T-2 toxin to rats, guinea pigs, and other experimental animals induced shock, hypothermia, and death due to cardiovascular and respiratory failure (Sato et al, 1975, Weaver et al., 1978, Feuerstein et al., 1985). In addition, cats that received intravascular (IV) or intraperitoneal (IP) administration of a single dose of T-2 toxin with 2 mg/kg induced a steady decline in mean arterial blood pressure (MBP) and pulse pressure to extreme shock level resulting in death after 5 to 15 h (Borison and Goodheart, 1989). These literatures showed an inconsistence of blood pressure (BP) responses following the toxin administration. On the other hand, deoxynivalenol (DON) that is also a representative mycotoxin of the trichothecene group (type B) has been widely known as a toxin that induces vomiting. The findings mentioned above suggest a possibility that DON which belongs to the same group with T-2 toxin may possess the similar cardiovascular responses to those by T-2 toxin. However, there is little evidence on cardiac effects of DON, although cardiac lesions combined with calcified pericarditis were caused by the ingestion of a diet containing 10 to 20 ppm of DON for a few weeks (Robbana-Barnat et al., 1987). Therefore, it is necessary to clarify whether DON has

hazardous effects on cardiac function through a detailed investigation.

It has been assumed that some differences in cardiovascular responses exist between acute and subchronic or chronic toxicity, in addition to the difference due to the administration route of DON or T-2 toxin. It is of importance to know both acute and subacute/chronic changes by these toxins for considering their toxic properties. In the present chapter, therefore, the acute circulatory changes were observed in anesthetized rats with administration of DON or T-2 toxin as the first step of studies. For this aim either T-2 toxin or DON was given in a single dose via subcutaneous route and the ECG and blood pressure parameters were observed during 3 h.

2.2 Materials and Methods

Animal and housing

Experiments were performed using 12-week old male Wistar rats weighing 260-280 g which were purchased from Japan SLC (Shizuoka, Japan). Animals were housed at 23±2 ℃ under a 12-h light/dark cycle (light on at 08:00 h) and were allowed free access to food and water. All experiments were conducted in accordance with the Animal Experimentation Guidelines of the University of Tokyo and approved by the institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences at the University of Tokyo.

Surgical Procedure and recordings of ECG and blood pressure

All rats were anesthesized with intraperitoneal administration of urethane (1-2 g/kg). Each rat was placed in a supine position and the trachea was cannulated with a

polyethylene tube. The open end of the tracheal cannula was connected to an artificial ventilator and the rat was ventilated at a rate of 80 breaths/min. This technique was performed in order to maintain the condition of the rat and to keep the spontaneous breathing. The femoral artery was catheterized by a polyethylene tube (Hibiki, No. PE-50) for continuous monitoring the systemic blood pressure. ECG was recorded according to the method described by Buschmann et al. (1980) using the standard limb lead II in the direction of the heart axis. The ECG electrodes were connected to an input box (ECG coupler) in series with a biological amplifier (180 system, San-ei Instrument Co., Tokyo) for recording the ECG. The systemic blood pressure was simultaneously recorded by the biological amplifier. The ECG and blood pressure signals were analyzed by using a software equipped with a data acquisition system (PowerLab 8/35, AD Instruments, Inc. Japan). After surgical preparation, rats were served as a control record for 10 min prior to the treatment with toxin administrations. Recordings of ECG and blood pressure were continuously performed before and during 3-h after the toxin administration.

Preparation and injection of toxins

DON and T-2 toxin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). T-2 toxin was dissolved in acetone/olive oil (5μl/1 ml), and the acetone was evaporated out under nitrogen gas for 30 minutes. DON was dissolved in 1 ml of propylene glycol.

Two separate experiments (Experiment I = DON exposure; Experiment II = T-2 toxin exposure) with identical conditions were conducted. In Experiment I, DON at doses of 0.5, 1 and 2 mg/kg was administered subcutaneously after 10 min of control

recording. In Experiment II, T-2 toxin at doses of 0.1, 0.5 and 1 mg/kg was injected in the same condition with DON experiment. Propylene glycol and olive oil was used as the control group in Experiment I and II, respectively.

Data analysis

ECG data were analyzed by the PowerLab software mentioned above. The ECG-wave components, i.e., PR interval, QRS duration, QT interval, as well as HR, were analyzed at 30-min intervals after the DON, T-2 toxin or vehicle injection. Furthermore, the ECG waveform and heart rhythm (R-R interval) were automatically or manually evaluated in order to detect episodes of arrhythmia before and after the DON or T-2 toxin administration.

Statistical analysis

All the results were statistically evaluated by two-way repeated-measures analysis of variance (two-way repeated-measures ANOVA) among all groups before and after vehicle, DON or T-2 toxin injections. The occurrence of arrhythmia was evaluated by one-way non-repeated-measures analysis of variance (one-way non-repeated-measures ANOVA). In addition, Fisher's test was used to test significant differences between control and treatment groups for all parameters. The data showing p values less than 0.05 were regarded as significant difference.

2.3 Results

Systolic Blood Pressure (SBP)

Changes in SBP could be observed at 30 to 180 min following to every dose of DON administration. A significant increase was identified in 0.5, 1 and 2 mg/kg-DON group compared with the control ($P<0.01$, two way repeated-measures ANOVA) as shown in Figure 2.1.

Figure 2.2 represents the significant increase of SBP started from 30 min following the administration of 0.5 and 1 mg/kg-T-2 toxin compared with the control ($P<0.01$ and 0.05). While 0.1 mg/kg-T-2 toxin induced a similar response with the vehicle-treated group. The SBP in 0.1 mg/kg-T-2 toxin group showed a significant difference compared with the control ($P<0.01$ and 0.05).

Diastolic Blood Pressure (DBP)

DON induced a significant increase of DBP in every dose of injection as shown in Figure 2.3. From 30 to 180 min, DBP in DON-treated groups were significantly higher than the control group ($P<0.01$). In the similar way, T-2 toxin at dose of 0.5 and 1 mg/kg caused a significant increase in DBP from 30 to 180 min compared with the control ($P<0.01$ and 0.05) (Figure 2.4). However in 0.1 mg/kg-T-2 toxin group did not show any significant change compared with the control.

Mean Arterial Blood Pressure (MBP)

Changes in MBP induced by DON and T-2 toxin are shown in Figure 2.5 and 2.6, respectively. DON increased MBP starting from 30 min after the administration.

Significant differences ($P<0.01$) in MBP were observed for both time-course and doses of DON injected. There were significant increases in MBP in the 0.5, 1 and 2 mg/kg-DON groups ($P<0.01$) from 30 to 180 min after the administration.

The administration of T-2 toxin increased MBP with no dose-dependent manner. The response was highest in the 0.5 mg/kg-T-2 toxin group ($P<0.01$ and 0.05) at 90 and 120 min after the administration. In the 1 mg/kg-T-2 toxin group, a significant increase of MBP ($P<0.01$ and 0.05) was also observed from 30 to 150 min after the administration. In the 0.1 mg/kg-T-2 toxin group, a significant decrease ($P<0.05$) in the MBP compared with that in the vehicle group was detected at 150 min after the administration.

Heart rate (HR)

The representative changes in HR that responded to DON and T-2 toxin administration are shown in Figure 2.7 and 2.8, respectively. DON decreased HR which started from 60 to 180 min in 0.5 and 1 mg/kg-DON group ($P<0.01$ and 0.05). While there was no significance identified in the 2 mg/kg-DON group, however the decreased trend of HR could be observed when compared with the vehicle administration group.

When 0.1 mg/kg-T-2 toxin was administered, HR was significantly declined from 30 to 180 min when compared with the vehicle administration group ($P<0.01$). While in 0.5 mg/kg-T-2 toxin group showed an increase trend of HR from 60 to 180 min as well as in 1 mg/kg-T-2 toxin group ($P<0.01$ and 0.05).

PR interval

Changes in PR interval are shown in Table 2.1. In 0.5 mg/kg-DON group, the PR interval was significantly prolonged from 30 to 90 min ($P<0.01$ and 0.05). The PR interval showed a significant increase from 60 to 180 min after 1 mg/kg-DON administration ($P<0.01$ and 0.05). In 2 mg/kg-DON group, the prolongation was observed only at 60 min after administration ($P<0.01$). After 60 min, without significant difference, the shortening of PR interval was observed in 2 mg/kg-DON group. T-2 toxin administration did not induce any significant changes in PR interval, although in the 1 mg/kg-T-2 toxin group the PR interval tended to be shortened after the T-2 toxin administration (Table 2.2).

QRS duration

Changes in QRS duration following DON and T-2 toxin were represented in Table 2.3 and 2.4, respectively. In 0.1 and 0.5 mg/kg-DON group, the QRS duration was significantly prolonged from 120 to 180 min after the administration ($P<0.01$ and 0.05). In 2 mg/kg-DON group, no significant difference was present in any time points compared with the control group.

T-2 toxin induced a prolongation of QRS duration in all doses of injection, where a significant increase was observed from 30 to 150 min as compared with the vehicle group ($P<0.01$ and 0.05).

QT interval

Changes in QT interval observed after DON- and T-2 toxin-administration are shown in Table 2.5 and 2.6, respectively. DON induced-QT interval prolongation could

be identified from 0.5-2 mg/kg-DON group. In 0.5 and 1 mg/kg-DON group, QT interval was significantly increased from 30 to 180 min ($P<0.01$ and 0.05). In 2 mg/kg-DON group, this significant change was identified from 120-150 min after administration ($P<0.01$). T-2 toxin induced a prolongation of QT interval from 30 to 150 min following the administration of this toxin in 0.5 and 1 mg/kg-T-2 toxin group, where the QT interval was significantly increased as compared with the vehicle injected-group ($P<0.01$).

R wave-amplitude

Changes in the R wave-amplitude observed after DON- and T-2 toxin-administration are represented in Figure 2.9 and 2.10, respectively. DON injection caused an increase in the R wave-amplitude in each dose of treatment. During 30 min to 180 min after the administration, the R wave-amplitude showed the significantly differences compared with the vehicle group ($P<0.01$). In contrast, T-2 toxin with a dose of 1 mg/kg reduced the R wave-amplitude at 90 to 180 min after the administration with no significant differences compared with the control group.

Arrhythmia

The administrations of DON and T-2 toxin induced arrhythmia that was represented by the premature ventricular contraction (PVC) and short-run type of ventricular tachycardia (Table 2.7 and 2.8). The PVC was found in 1 and 2 mg/kg-DON administration groups, however no significant occurrences were found when compared with vehicle administration group. In a similar way, PVC was observed in both vehicle and T-2 toxin injection groups but no significant occurrences were identified. The

short-run type of ventricular tachyarrhythmia appeared characteristically in 0.1 and 0.5 mg/kg-T-2 toxin administration group, especially, in the 0.5 mg/kg-T-2 toxin group a significant difference of occurrence was found when compared with the vehicle administration group ($P<0.05$).

2.4 Discussion

In the present study, acute effects of DON and T-2 toxin on the cardiovascular system in rats with via subcutaneous injection of toxins under anesthetic condition were examined. The administration of both DON and T-2 toxin immediately developed an increase of SBP, DBP and MBP. This finding is consistent with a previous report (Feuerstein et al., 1985) in which hypertension induced by T-2 toxin (0.5-2 mg/kg, IV) was found immediately after the injection and had prolonged for 6-8 h. The decrease in HR, in the present study, was observed after 90 min following the toxin administration in accordance with the increase in MBP in the DON administration. This change in the HR is considered as the baroreceptor reflex that might continuously happen since the toxin had not been completely eliminated and still existed in the body during the term of observation. Thus the HR is regulated by the vagal reflex pathway in response to the elevation of systemic blood pressure. However, this reflex phenomenon was observed only in DON administration and could not be observed in T-2 toxin administration. A possibility is assumed that the toxic effect of T-2 toxin inhibits the normal baroreceptor reflex or T-2 toxin has a direct action to the heart to activate a firing rate at the sinus node that leads to the increase of HR.

When considered the ECG-waveform, DON induced a minor extent of prolongation of the QRS duration that is a parameter of ventricular depolarization. Such a change might reflect weak toxic effects of DON on the ventricular myocardium. The shortening of QRS duration following T-2 toxin in this study might be partly associated with an increase in HR (shortening of the RR interval) that is influenced by an increase of sympathetic tone on the sinus node and enhances ventricular conduction (Goldberger and Bhargava, 1983).

The QT interval provides a measure of ventricular repolarization and is determined by the balance of the repolarizing inward sodium and calcium currents, and the outward potassium and chloride currents. The QT prolongation was observed in the 0.5 and 1 mg/kg-T-2 toxin administration groups. Such a change in the QT interval may reflect some dysfunctions of ventricular repolarization process that relates to a voltage-gated potassium channels. As London et al. (1998) reported that the mutations of K^+ channel genes have been shown to cause arrhythmias and sudden death in families with the congenital long QT syndrome. It is feasible that T-2 toxin might disrupt ventricular repolarization at the voltage-gated potassium channel and caused the QT prolongation in the present study. Furthermore, QT interval correlates with measurements of cardiac autonomic function as shown in type 2 diabetic patients who had the cardiac vagal dysfunction showed a sign of QT prolongation (Takahashi et al., 2004). Though the autonomic nervous activity has not been evaluated in this chapter, it has a possibility that the QT prolongation observed in the acute experiments in the present study might receive an influence from both the disturbance of the repolarization process and the autonomic nervous defects induced by T-2 toxin.

The increase of R wave-amplitude has been usually thought to reflect the cardiac enlargement, cardiac congestion, and/or cardiac hypertrophy. The marked increase in the R wave-amplitude in all doses of DON administration in the present study suggested that the volume overload significantly increased in the heart during 3 h at least after the administration. However, the myocardial contractility was assumed to be maintained at normal or higher level compared to that in the pre-administration of DON. On the other hand, the small extent of the reduction of R wave-amplitude by T-2 toxin may reflect the tendency for myocardial contractility in the present study. This finding is not inconsistent with the previous report, i.e., Yarom *et al.* (1986) reported that T-2 toxin with a dose above 250 μ g/ml decreased the beat rate and amplitude of action potential in cultured myocardial cells. Though this study by Yarom et al. was performed through *in vitro* experiments, the analogous response with decreased amplitude of R wave by the administration of T-2 toxin was recognized in the present study.

It was evidenced that T-2 toxin induced the short-run type of ventricular tachycardia in this study. This type of arrhythmia is considered as an inducing factor for ventricular tachyarrhythmia and ventricular fibrillation that are major causes of the sudden death in humans. Such cardiac abnormalities may be a risk factor to increase in severity in some patients with cardiac and/or metabolic disorders if the amount of toxin is over the normal level of detoxification in the living body.

In conclusion, in the present study the acute toxicity via subcutaneous administration of DON and T-2 toxin on the cardiovascular system has been clarified. These trichothecene mycotoxins might cause a direct toxicity to the cardiovascular system and the autonomic nervous system (ANS). In the following chapter, the toxicity to ANS is mainly investigated in order to elucidate the mechanisms of the

trichothecene mycotoxins in causing cardiac function impairment.

2.5 Summary

DON and T-2 toxin, representative trichothecene mycotoxins, developed significant alteration in SBP, DBP, MBP, HR, and ECG-waveform with the occurrence of arrhythmia during 3 h under the anesthetic condition. The results in this chapter clarified that these mycotoxins have a potent cardiac toxicity following subcutaneous administration.

Table 2.1. PR intervals in the control and DON-groups

	Time (min) after administration with vehicle (control) or DON (0.5-2 mg/kg)						
	0	30	60	90	120	150	180
Propylene glycol (n=5)	43.56±1.70	47.90±2.49	49.05±2.63	53.43±5.84	53.79±6.47	53.81±6.22	55.54±9.04
0.5 mg/kg (n=6)	54.07±1.61	55.77±1.07*	58.47±1.60**	60.26±2.03*	56.88±1.77	56.07±1.94	55.24±1.90
1 mg/kg (n=6)	53.73±3.56	51.94±2.63	56.79±2.04*	63.93±3.81**	60.64±2.07*	76.92±11.43**	69.25±15.27**
2 mg/kg (n=5)	57.94±10.61	52.62±2.73	59.33±5.77**	53.81±2.93	54.80±3.59	55.44±4.13	52.70±3.67

The asterisks show significant differences at $P<0.01$ (**) and 0.05 (*) from the control value at each time point.

Zero min is the time before the vehicle- or DON-injection. Data are expressed as the mean ± S.E.

Table 2.2. PR intervals in the control and T-2 toxin-groups.

Time (min) after administration with vehicle (control) or T-2 toxin (0.1-1 mg/kg)							
	0	30	60	90	120	150	180
Olive oil (n=4)	43.93±1.33	45.57±1.19	46.72±1.45	45.28±1.33	42.54±1.50	41.77±1.79	40.74±1.66
0.1 mg/kg (n=6)	50.55±1.94	50.51±2.17	51.65±2.20	51.57±2.24	51.86±1.99	51.59±2.13	50.98±1.96
0.5 mg/kg (n=4)	46.08±7.96	46.48±1.04	46.81±1.13	46.50±1.22	45.52±9.42	44.44±6.79	43.57±6.39
1 mg/kg (n=4)	47.12±1.71	45.43±5.69	45.01±6.36	44.36±8.74	42.55±1.21	41.31±1.14	39.81±9.54

No significance was detected after T-2 toxin administration.

Table 2.3. QRS duration in the control and DON-groups

	Time (min) after administration with vehicle (control) or DON (0.5-2 mg/kg)						
	0	30	60	90	120	150	180
Propylene glycol (n=5)	14.00±1.2	13.77±1.22	14.23±1.48	15.15±2.41	15.22±2.48	15.05±2.49	14.87±2.37
0.5 mg/kg (n=6)	16.8±1.71	16.39±1.13	16.56±9.53	16.56±1.07	18.61±1.99*	18.5±2.10**	18.06±1.93*
1 mg/kg (n=6)	16.96±1.95	16.53±1.29	16.93±1.34	17.58±1.21	17.93±1.22	18.08±1.34	19.18±1.53*
2 mg/kg (n=5)	16.3±1.16	16.23±1.36	16.72±1.43	17.12±1.52	17.23±15.75	17.28±1.57	17.32±1.78

The asterisks show significant differences at P<0.01 (**) and 0.05 (*) from the control value at each time point. Zero min is the time before the vehicle- or DON-injection. Data are expressed as the mean ± S.E.

Table 2.4. QRS duration in the control and T-2 toxin-groups

	Time (min) after administration with vehicle (control) or T-2 toxin (0.1-1 mg/kg)						
	0	30	60	90	120	150	180
Olive oil (n=5)	16.35±1.61	13.73±0.64	13.93±0.73	14.87±1.22	15.45±1.68	15.3±1.86	15.52±2.07
0.1 mg/kg (n=6)	14.71±0.88	14.07±1.02	15.06±0.98**	15.67±1.11*	15.56±1.16	15.47±1.26	15.22±1.20
0.5 mg/kg (n=6)	13.8±0.57	13.60±0.50**	13.31±0.49	13.15±0.54	13.13±0.51	13.32±0.59	12.86±0.36
1 mg/kg (n=5)	12.35±1.88	12.38±0.84**	12.6±0.55**	12.38±0.54**	12.1±0.55*	12.02±0.58*	12.02±0.73

The asterisks show significant differences at P<0.01 (**) and 0.05 (*) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean ± S.E.

Table 2.5. QT intervals in the control and DON-groups

	Time (min) after administration with vehicle (control) or DON (0.5-2 mg/kg)						
	0	30	60	90	120	150	180
ylene glycol (n=5)	66.21±2.89	72.91±6.66	73.46±5.70	76.88±8.11	73.60±7.05	74.07±7.57	73.59±7.3
ng/kg (n=6)	92.06±4.19	90.87±3.54**	97.87±3.51**	99.49±4.62**	101.36±1.23**	100.72±5.43**	101.22±3.66
g/kg (n=6)	83.08±7.93	86.90±7.07**	94.65±9.57**	98.25±9.83**	101.81±12.01**	105.18±11.25**	110.63±11.0
g/kg (n=5)	73.31±7.63	75.47±7.91	78.71±8.02	83.21±8.31	85.55±7.59**	88.88±5.67**	89.80±6.56

The asterisks show significant differences at P<0.01 (**) and 0.05 (*) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean ± S.E.

Table 2.6. QT interval in the control and T-2 toxin-groups

	Time (min) after administration with vehicle (control) or T-2 toxin (0.1-1 mg/kg)						
	0	30	60	90	120	150	180
Olive oil (n=3)	83.22±3.46	76.04±7.45	73.56±7.31	71.79±7.67	66.50±10.3	65.67±7.62	64.47±5.93
0.1 mg/kg (n=2)	78.74±8.28	86.61±6.94	84.26±7.70	86.79±8.48	86.75±9.52	84.7±11.39	80.75±11.65
0.5 mg/kg (n=4)	79.82±4.59	79.53±3.43**	85.75±2.37**	88.02±2.92**	88.26±3.35**	87.39±3.09**	84.54±1.60**
1 mg/kg (n=3)	67.61±7.61	73.21±7.33**	74.94±6.80**	74.56±7.21**	73.79±6.91**	72.62±6.21**	72.52±6.05

The asterisks show significant differences at $P<0.01$ (**) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean ± S.E.

Note: QT interval could not be measured in some points of time, thus the sample sizes decreased. The data with a sample size less than 3 was not used for statistical analysis.

Table 2.7. Occurrence of arrhythmias in the control and DON-groups

Arrhythmia	**Dose (mg/kg)**			
	0	**0.5**	**1**	**2**
Pre-mature ventricular contraction	0/5(0)	0/6(0)	2/6(0.5)	2/5(0.4)

No significant difference of arrhythmic occurrence was observed within 3 hourse following DON-administration when compared with the control. No evidence of this arrhthymia appeared in the 0-mg/kg DON group (vehicle). In the data, the occurrence of arrhythmia was calculated as an amount of animal in which the representative arrhythmia was identified (n=5 or 6/group). The average of occurrence of arrhythmia in each rat was represented in the parentheses. DON: Deoxynivalenol.

Table 2.8. Occurrence of arrhythmias in the T-2 toxin-groups

Arrhythmia	**Dose (mg/kg)**			
	0	**0.1**	**0.5**	**1**
PVC	2/5 (2.17)	3/6 (2.16)	3/6 (0.67)	1/5(1)
Short-run	0/5 (0)	1/6 (0.17)	3/6 (0.5)*	0/5 (0)

A significant increase of arrhythmia was observed for short-run in 0.5 mg/kg-T-2 toxin group (*P<0.05). In the data, the occurrence of arrhythmia was calculated as an amount of animal in which the representative arrhythmia was identified (n=5 or 6/group). The average of occurrence of arrhythmia in each rat was represented in the parentheses. Arrhythmias are presented: PVC: premature ventricular contraction; Short-run: short-run type of ventricular tachycardia.

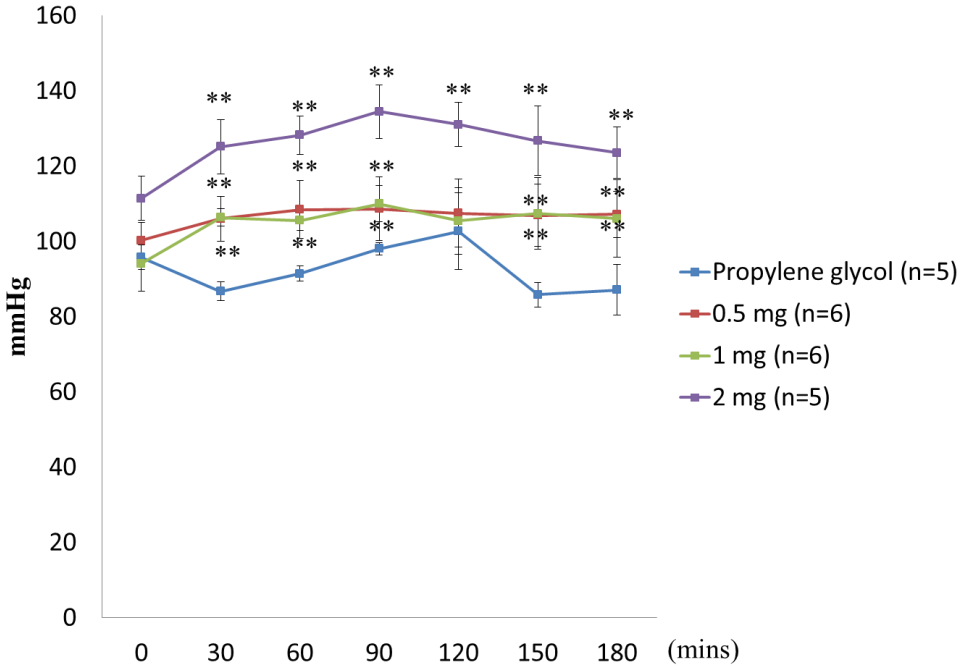


Figure 2.1. Changes in systolic blood pressure (SBP) recorded from the femoral artery in response to DON-injection. The asterisks show significant differences at P<0.01 (**) from the control value at each time point. Zero min is the time before the vehicle- or DON-injection. Data are expressed as the mean ± S.E.

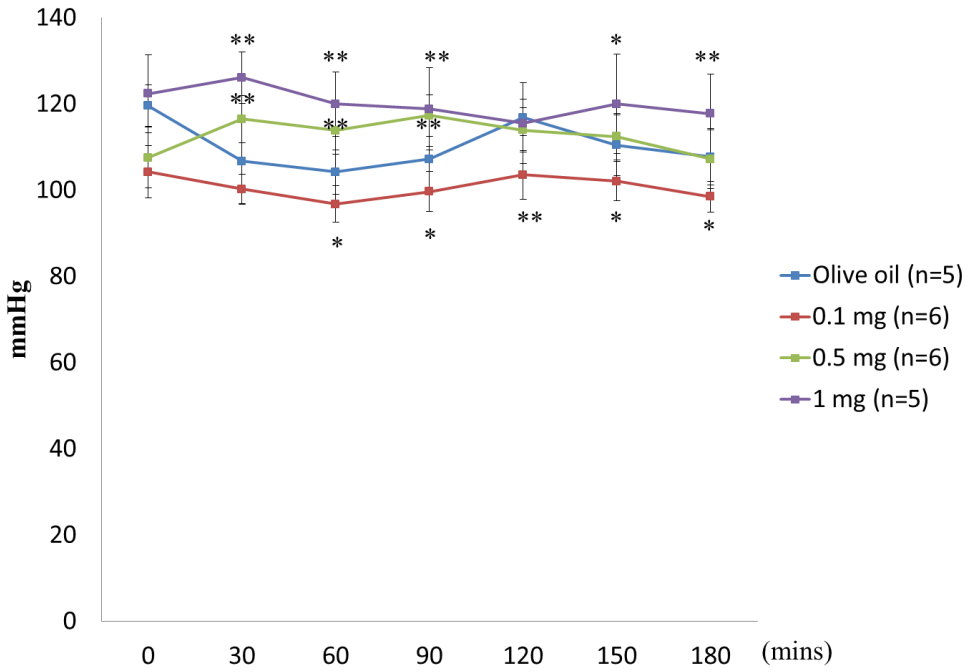


Figure 2.2. Changes in systolic blood pressure (SBP) recorded from the femoral artery in response to T-2 toxin-injection. The asterisks show significant differences at P<0.01 (**) and 0.05 (*) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean ± S.E.

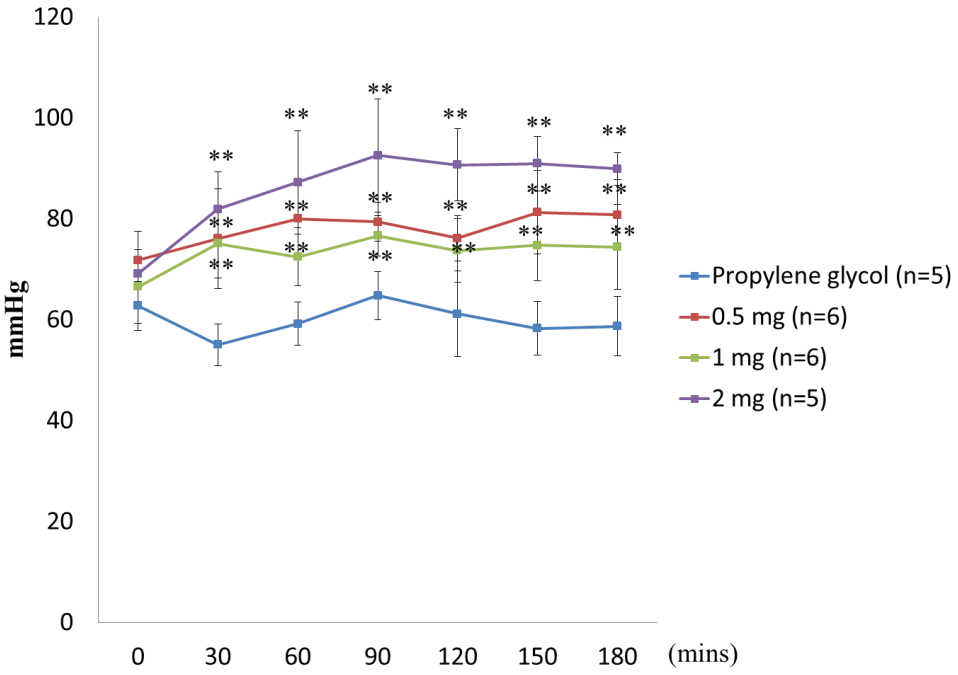


Figure 2.3. Changes in diastolic blood pressure (DBP) recorded from the femoral artery in response to DON-injection. The asterisks show significant differences at P<0.01 (**) from the control value at each time point. Zero min is the time before the vehicle- or DON-injection. Data are expressed as the mean ± S.E.

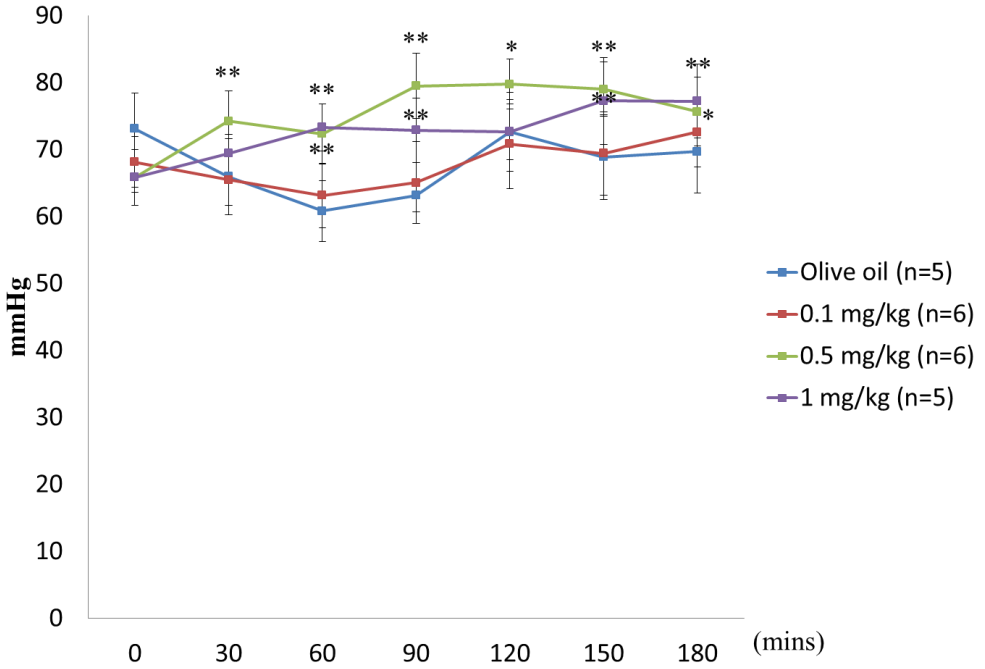


Figure 2.4. Changes in diastolic blood pressure (DBP) recorded from the femoral artery in response to T-2 toxin-injection. The asterisks show significant differences at P<0.01 (**) and 0.05 (*) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean ± S.E.

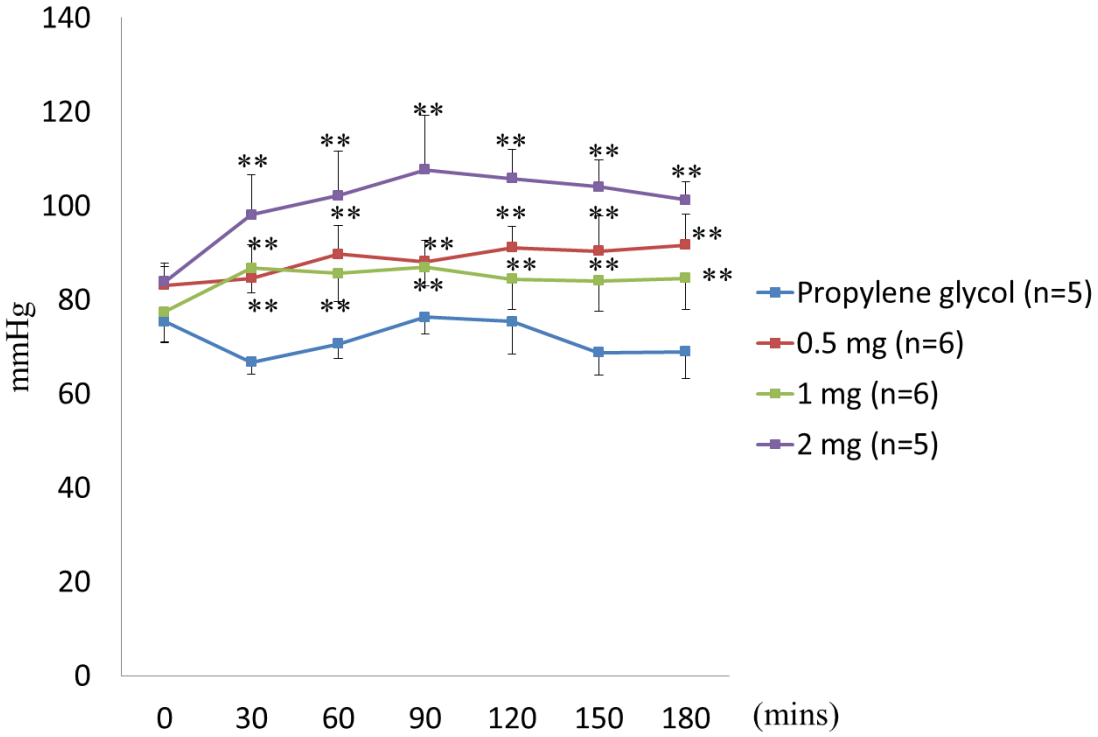


Figure 2.5. Changes in mean arterial blood pressure (MBP) recorded from the femoral artery in response to DON-injection. The asterisks show significant differences at P<0.01 (**) from the control value at each time point. Zero min is the time before the vehicle- or DON-injection. Data are expressed as the mean ± S.E.

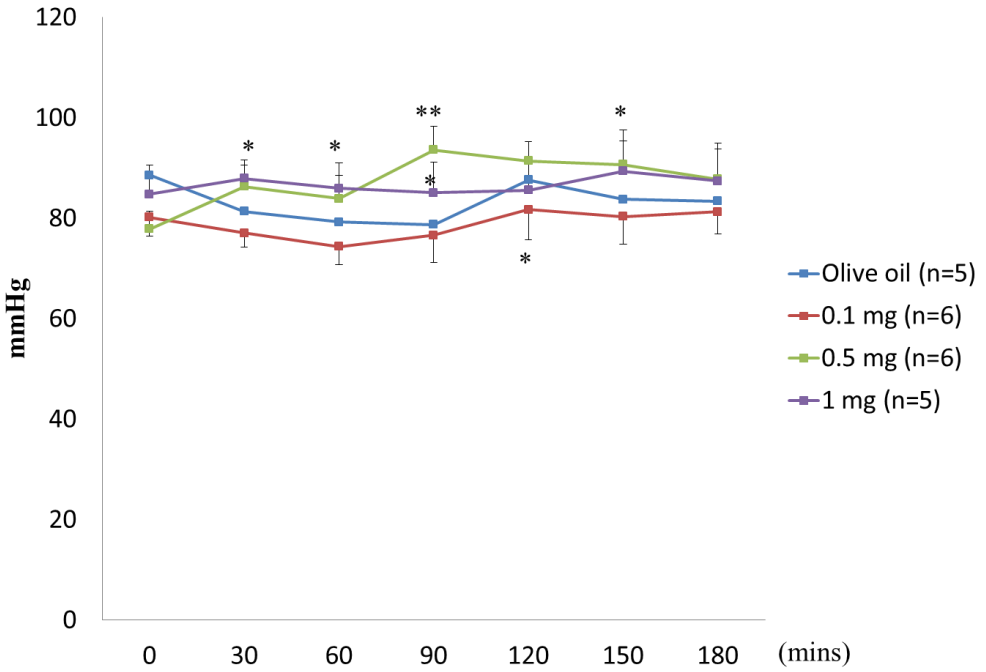


Figure 2.6. Changes in mean arterial blood pressure (MBP) recorded from the femoral artery in response to T-2 toxin-injection. The asterisks show significant differences at P<0.01 (**) and 0.05 (*) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean ± S.E.

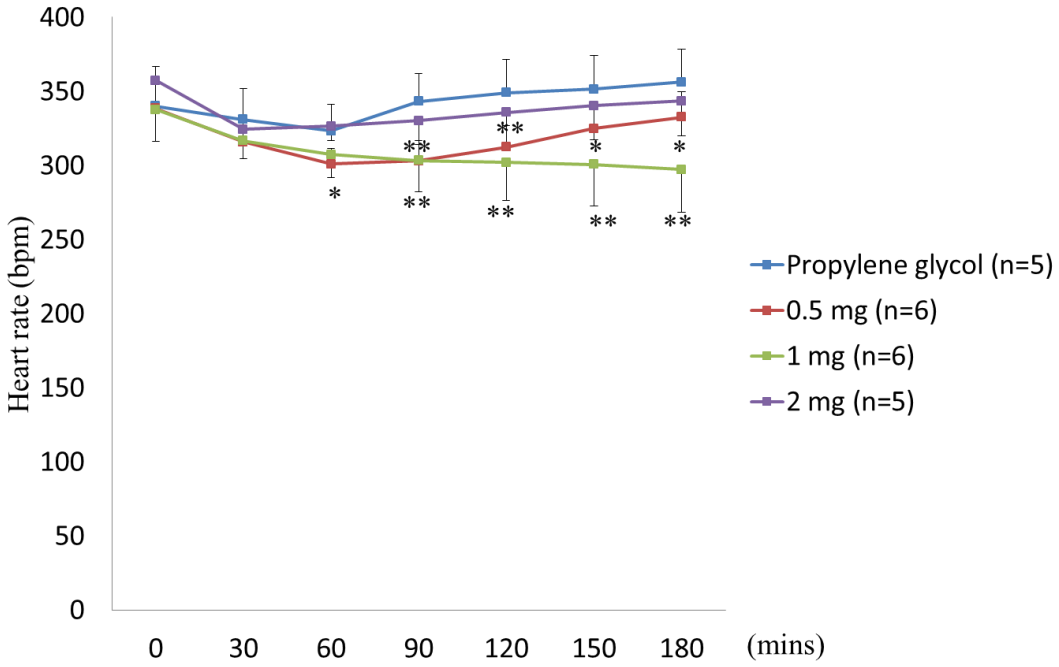


Figure 2.7. Changes in heart rate (HR) in response to DON-injection. The asterisks show significant differences at P<0.01 (**) and 0.05 (*) from the control value at each time point. Zero min is the time before the vehicle- or DON-injection. Data are expressed as the mean ± S.E.

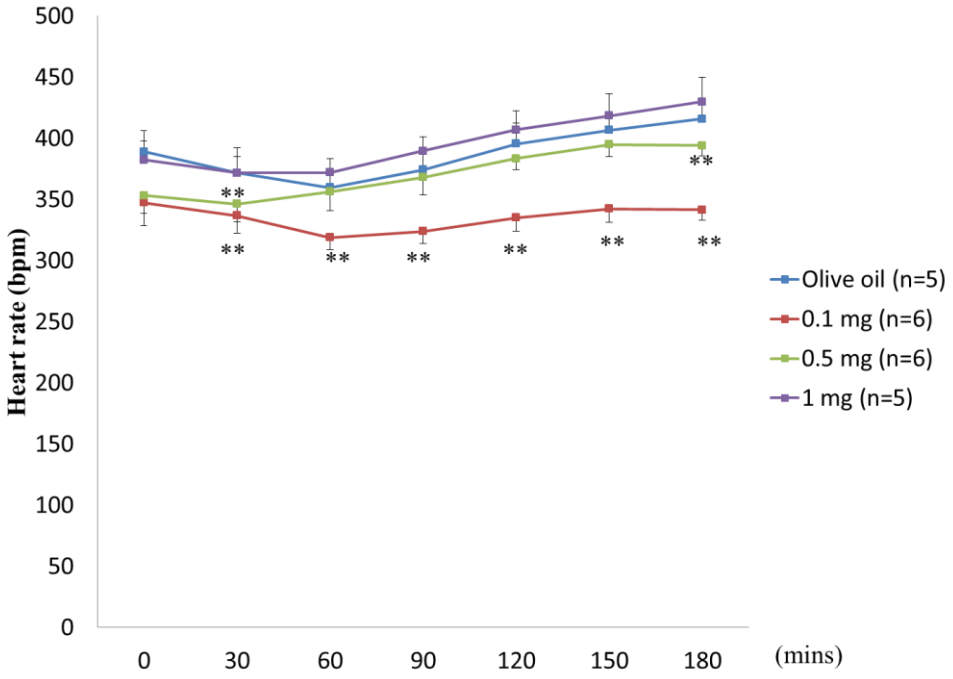


Figure 2.8. Changes in heart rate (HR) in response to T-2 toxin-injection. The asterisks show significant differences at P<0.01 (**) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean ± S.E.

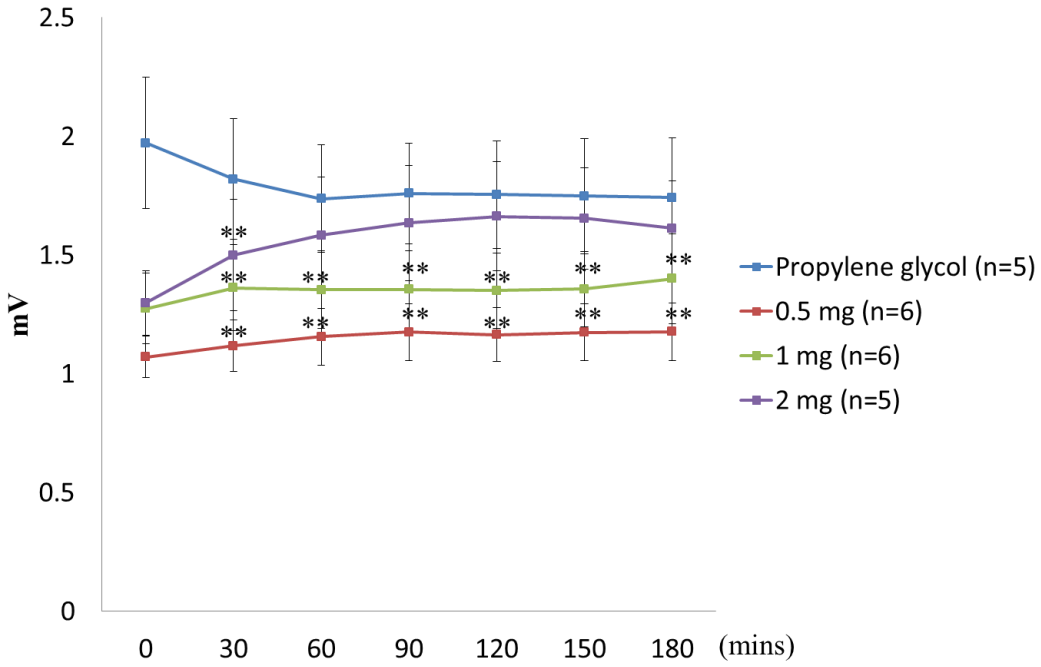


Figure 2.5. Changes in R wave-amplitude in response to DON-injection. The asterisks show significant differences at P<0.01 (**) from the control value at each time point. Zero min is the time before the vehicle- or DON-injection. Data are expressed as the mean ± S.E.

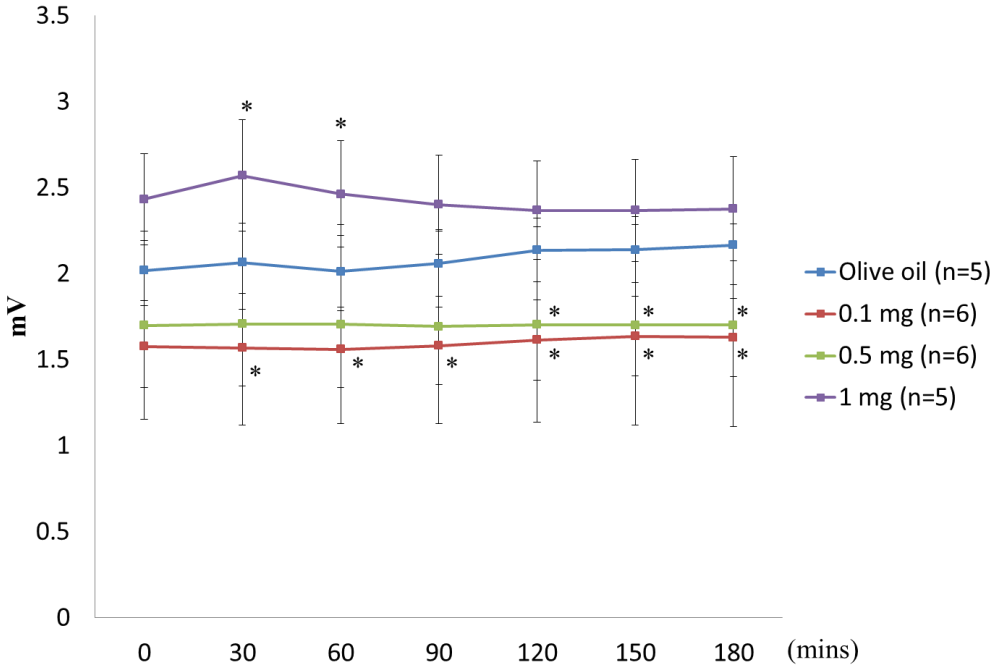


Figure 2.6. Changes in R wave-amplitude in response to T-2 toxin-injection. The asterisks show significant differences at $P<0.05$ (*) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as the mean $\pm$ S.E.

CHAPTER 3

ARRHYTHMIAS AND ALTERATIONS OF THE AUTONOMIC NERVOUS ACTIVITY INDUCED BY TRICHOTHECENE MYCOTOXIN THROUGH TELEMETRIC MEASUREMENTS IN UNRESTRAINED RATS

3.1 Introduction

From Chapter 2, the findings suggested that the trichothecene mycotoxin possesses a high possibility of cardiac toxicity in its nature. However, the previous studies including the study in Chapter 2 were performed only under the acute stage of response observed mostly for a few or several hours in anesthetized animals or *in vitro* experiments, and there is little evidence about precise changes in cardiac and autonomic nervous activity for longer periods with consecutive recordings in conscious animals. Therefore, it is considered to be important that changes in the cardiac function and autonomic nervous activity are observed for longer period in unrestrained and conscious rats.

In this chapter, the heart rate variability (HRV) on the basis of fast Fourier transform (FFT) was used to analyze the autonomic nervous activity, where the HRV reflects the relative activity of the sympathetic and parasympathetic nervous system in rats with administration of DON and T-2 toxin.

Furthermore, the pharmacological treatment of the autonomic nervous system, i.e., pretreatments with atropine or propranolol, was performed to assess the participation of the sympathetic and parasympathetic nervous activity to cardiac functions in rats with those mycotoxins. These experiments were thought to be important to evaluate whether the cardiac changes produced by each mycotoxin were originated from the intrinsic myocardial function or the autonomic nervous activity.

The aim of this study was to elucidate the toxic effects of DON and T-2 toxin on cardiac and autonomic nervous functions through longer observation in unrestrained and conscious rats by a telemetric recording method and by use of pharmacological

manipulations of the autonomic nervous system.

2.2 Materials and Methods

Animals and housing

All the experiments were performed using 49 male Wistar rats purchased from Japan SLC, Inc. (Shizuoka, Japan) at 8 weeks of age and having the body weights of 230-250 g at 10 weeks of age when the telemetric device was implanted. Each rat was maintained with *ad libitum* access to food and water in an individual cage within an isolation chamber maintained under controlled lighting (light-dark cycle, light=12:00-24:00, dark=24:00-12:00) and temperature conditions (24^{o}C). All rats were fully adapted to these breeding environments during experiments.

Implantation of Telemetry Device for Electrocardiogram (ECG) Recording

Each rat underwent one-week adaptation period before the surgical operation. Then, a small telemetry device (weight=3.9g, volume=1.9cc; TA10ETA-F20, Data Sciences International, St. Paul, MN) for transmitting ECG was implanted into the dorsal subcutaneous region under systemic anesthesia with 30 mg/kg (IP) administered pentobarbital sodium. Paired wire electrodes that came with the telemetry device were placed under the skin of the dorsal and ventral thorax to record the apex-base (A-B) lead ECG.

ECG signal acquisition and data analysis

One week after the surgery for implantation of the telemetry device, ECG signals

were recorded from each rat in a cage that had been placed on a signal-receiving board (RA 1610, Data Sciences International, St. Paul, MN). The ECG data was sampled continuously at 1-msec intervals, and all the data analyses, including fast Fourier transform (FFT) analysis and ECG wave component analyses, were performed using an ECG processor analyzing system (SBP2000U, Softron, Tokyo, Japan) equipped with a personal computer in series with an analog-digital converter; the ECG data were stored on an external hard disk.

The ECG wave components, i.e., PR interval, QRS duration, QT interval, and HR, were analyzed for each 3 days before and after T-2 toxin or vehicle injection. In case of DON, data were analyzed for 1 day before and after DON or vehicle injection. Furthermore, the ECG waveform and heart rhythm (RR interval) were automatically or manually evaluated in order to detect episodes of arrhythmia before and after DON or T-2 toxin administration.

In addition, the autonomic nervous function analyzed by heart rate variability (HRV) was evaluated using software (SRV2W, Softron, Tokyo, Japan) on a personal computer through which the power spectrum was obtained by FFT analysis of the frequency component of the RR interval on ECG based on the Cooley-Tukey FFT algorithm (Cooley and Tukey, 1965). In rats, there are two major spectral components, i.e., the low frequency (LF) (0.1-1.0 Hz) and high frequency (HF) (1.0-3.0 Hz) components, in the power spectrum analysis. A previous study using autonomic nervous blockades indicated that the LF component was influenced by both sympathetic and parasympathetic nervous activity and that the HF component was affected only by the parasympathetic nervous activity. Accordingly, the LF/HF ratio indicated the balance between the sympathetic and parasympathetic nervous activity (Kuwahara et al., 1994).

Toxin preparation

The preparation of DON and T-2 toxin were performed in accordance with the description in Chapter 2.

Injection protocol

All rats were randomly assigned for two groups with administration of DON (Experiment I) or T-2 toxin (Experiment II). In Experiment I, DON at a dose of 0.5, 1 or 2 mg/kg was subcutaneously administered to each rat 3 days after the control injection. In Experiment II, T-2 toxin at a dose of 0.1 or 0.5 mg/kg was injected in the same condition as in the DON experiment. The subcutaneous injection route was selected in the present study because the aim of the study was to observe cardiac and autonomic nervous responses to longer action of T-2 toxin, because subcutaneous injection of this toxin has been known to result in slow absorption through the skin, and because some tissue damages caused by oxidative stress on the other factors were shown to gradually increase to the maximum level at the third day after the injection (Chaudhary & Lakshmana, 2010). Furthermore, the trichothecene group of mycotoxins usually produces strong gastrointestinal inflammation, anorexia, and in some animal species, vomit (Pang V. F. et al., 1987; Borison & Goodheart, 1989), which may mask or interfere with the toxic nature of the mycotoxin in the extra-digestive organs. All injections were performed at 12:00 when the light period started, on each injection day. This injection time was selected to avoid large disturbances in biorhythm resulting from handling stress due to injections in midway through the light or dark period. The doses of DON and T-2 toxin in the present study were chosen by referring to the past reports on the cardiac effects of T-2 toxin (Feuerstein et al., 1985; Magnuson et al., 1987; Wang

et al., 1998).

T-2 toxin at the doses of 0.1 (n=6) and 0.5 (n=5) mg/kg and DON at the doses of 0.5 (n=6), 1 (n=6) and 2 (n=6) mg/kg were subcutaneously injected twice at an interval of 3 days, to the rats. Furthermore, 0.2 ml of olive oil and propylene glycol served as a vehicle control was given to the rats at 3 days before the administration of T-2 toxin and DON, respectively, in all experimental groups. In the present study, the cardiac effects of a higher dosage of T-2 toxin, e.g., 1 mg/kg, were not described because of early fatal termination during observation after administration.

Effects of the Autonomic Nerve Blockades

Effects of atropine

Atropine sulfate (Tokyo Chemical Industry Co., Ltd.) dissolved into 0.9% NaCl-10mM HCl (Carre et al., 1994) was used to observe the effects of parasympathetic blockade on the ECG changes caused by T-2 toxin and DON. All rats in this group received atropine infusion through an osmotic minipump (model 2ML1, Alzet Osmotic Pumps, DURECT Corporation, Cupertino, CA, U.S.A.) at a rate of 20 mg/kg/day for 7 consecutive days. Before osmotic minipump implantation, 0.2 ml of olive oil and propylene glycol was given as a control for T-2 toxin and DON, respectively. At the third day after implantation of the osmotic minipump, 0.5 (n=5) mg/kg of T-2 toxin and 2 (n=5) mg/kg of DON was administered to each rat in this group. No repeated injection of toxin was performed in this group.

Effects of propranolol

Propranolol hydrochloride (Tokyo Chemical Industry Co., Ltd.) dissolved into

0.9% NaCl-10mM HCl (Carre et al., 1994) was used to observe the effects of sympathetic blockade on the ECG changes caused by T-2 toxin and DON. All rats in this group received propranolol infusion through an osmotic minipump (model 2ML1, Alzet) at a rate of 100 mg/kg/day for 7 consecutive days. At the third day after implantation, 0.5 (n = 5) mg/kg of T-2 toxin and 2 (n = 5) mg/kg of DON was administered to this group. No repeated injection of toxin was performed in this group. As described above in the experiment with atropine, 0.2 ml of olive oil and propylene glycol served as T-2 toxin and DON, respectively a control and were given before propranolol infusion.

Statistical Analysis

All the results were statistically evaluated by two-way repeated-measures analysis of variance (two-way repeated-measures ANOVA) among all groups before and after vehicle or T-2 toxin and DON injections. Comparisons between the T-2 toxin, "T-2 toxin + atropine," and "T-2 toxin + propranolol" groups were firstly performed with systemic random sampling and statistically evaluated by one-way non-repeated-measures analysis of variance (one-way ANOVA without replication) and two-way non-repeated-measures analysis of variance (two-way ANOVA without replication). In DON experiment, the comparisons were performed in the similar way with T-2 toxin experiment. In addition, Fisher's test was used to test significant differences between control and treatment groups for PR interval, QRS duration, QT interval, the occurrence of arrhythmia, and HR and HRV values. Differences with p values less than 0.05 were regarded as significant.

2.3 Results

Heart rate (HR) changes in short-term observation

Short-term changes in HR were observed before and immediately after the DON-injection, as shown in Figure 3.2. Significant differences ($P<0.05$, Two-way repeated-measures ANOVA) were observed for both time and doses of DON injected. There were significant increases in HR in the 1.0 and 2.0 mg/kg-DON groups ($P<0.05$) from 90 to 150 min, while at 180 min, only the 2 mg/kg-DON group showed a significant difference ($P<0.05$). At 0, 30, or 60 min after DON administration, no significant differences were recognized between the control group and the DON-injected groups. Also, there was no significant difference in the 0.5 mg/kg-DON group at any time point.

Changes in HR by T-2 toxin administration were represented in Figure 3.3. From 90 to 180 min, T-2 toxin significantly raised the HR in the 0.1 and 0.5 mg/kg-T-2 toxin groups ($P<0.01$). No significant changes were identified between the control group and the T-2 toxin-administered groups at 0, 30 or 60 min after T-2 toxin administration, which was the similar time-course change in DON.

Heart rate (HR) changes in long-term observation and effects of blockers

The representative changes in HR that occurred during the telemetric ECG recording period are shown in Figure 3.1 and 3.4, and periodic alterations in HR that were dependent on the light-dark cycle were revealed. DON at dose of 1 mg/kg caused a significant increase of HR and this response lasted approximately one day and the overall HR returned to its normal state (Figure 3.1). While injection of T-2 toxin at

concentrations of 0.1 mg/kg and 0.5 mg/kg induced an increase in HR, which maintained a diurnal rhythm and the increased HR lasted for the two or three days observed after the first and second injections (Figure 3.4A and 3.4B).

HR changes by DON administration are demonstrated in Figure 3.5. As described above, the effect of DON on HR was exhibited within one day after the administration, thus all the data in DON experiment were analyzed for only one day after the administration. DON at dose of 0.5 and 1 mg/kg did not induce any significant changes in HR in both light and dark period (Figure 3.5A and 3.5B). However, 2 mg/kg of DON-administration significantly increased the HR at light period in the second injection ($P<0.05$) as represented in Figure 3.5C. On the basis of this finding, the dosage of 2 mg/kg of DON was adopted in the study on the alteration of HR in response to the autonomic nervous blockades, i.e. atropine and propranolol. The results are shown in Figure 3.5D. Atropine alone induced the increase in HR in both light and dark period as compared with HR in the control ($P<0.01$ and $P<0.05$) (Figures 3.5D). In the similar way, propranolol alone decreased HR in both the light and dark periods ($P<0.01$) (Figures 3.5D). As represented in Figure 3.5D, there was a significant change when compared between DON and DON + atropine at only light period ($P<0.01$). In addition, the comparison between DON and DON + propranolol, significances were identified in both light and dark period ($P<0.01$).

The changes in HR in response to the T-2 toxin administration are summarized in Figure 3.6. Significant differences ($P<0.05$, two-way repeated-measures ANOVA) in HR were observed for order and doses of T-2 toxin injection. There was a significant increase in HR in the 0.5 mg/kg T-2 toxin groups ($P<0.01$ or $P<0.05$; Figure 3.6B) during the 3 days of observation, while the 0.1 mg/kg T-2 toxin group did not show any

significant difference (Figure 3.6A). In the 0.5 mg/kg T-2 toxin group, HR significantly increased in the light period after both the first and second administrations ($P<0.01$) compared with the control, while in the dark period, only the first administration showed an increase ($P<0.05$). Also, the significant difference was found between the first and second administrations in both the light ($P<0.05$) and dark periods ($P<0.01$).

Comparing HR among the group with only T-2 toxin and two groups with autonomic nervous blockers, there was no significant change between T-2 toxin and T-2 toxin + atropine, while there was a significant decrease ($P<0.01$) in the comparison between the T-2 toxin and T-2 toxin + propranolol groups in both the light and dark periods (Figure 3.6C).

PR interval in short-term observation

The changes in the PR interval observed after the administration with vehicle or DON (0.5-2.0 mg/kg) are shown in Table 3.1A. No significant difference was observed in the 0.5 or 1.0 mg/kg-DON groups compared with the control. However, in the 2.0 mg/kg-DON group, a significant increase ($P<0.05$) in the PR interval compared with that in the control group was detected at 120 min.

T-2 toxin significantly induced PR prolongation which was observed at 90 and 150 min following the administration of 0.5 mg/kg-T-2 toxin ($P<0.01$ and $P<0.05$, respectively), whereas at 180 min PR interval become shorten when compared with the control group at doses of 0.1 and 0.5 mg/kg-T-2 toxin ($P<0.05$ and $P<0.01$, respectively) as shown in Table 3.1B.

PR interval in long-term observation and effects of blockers

DON caused no PR prolongation in long-term observation and no significant differences were found in any doses of treatment (Figure 3.7A).

The changes in the PR interval observed after the administration of vehicle or T-2 toxin (0.1 and 0.5 mg/kg) are shown in Figure 3.8A. A significant decrease was revealed in the 0.5 mg/kg T-2 toxin group ($P<0.05$), whereas the 0.1 mg/kg T-2 toxin group showed a significant increase in the second administration compared with that in the control ($P<0.05$).

When the animals were pretreated with the blockers, there were no significant differences between the atropine and control groups, whereas propranolol alone significantly prolonged the PR interval ($P<0.05$) (Figure 3.8D). The administration of the combination of T-2 toxin and atropine and the combination of T-2 toxin and propranolol significantly prolonged the PR interval ($P<0.0.5$ and $p<0.01$ respectively), as compared with T-2 toxin (0.5 mg/kg) alone (Figure 3.8D).

QRS duration in short-term observation

The changes in the QRS duration that occurred after vehicle- or DON-administration are shown in Table 3.2A. No significant difference was present in any treatment group compared with the control group.

T-2 toxin with doses of 0.1 mg/kg and 0.5 mg/kg significantly shortened the QRS duration at 150 min and 30 min, respectively, following administration ($P<0.05$) (Table 3.2B).

QRS duration in long-term observation and effects of autonomic nervous blockers

DON did not change QRS duration in longer-term observation for three days and no significant differences were found in any doses of treatment (Figure 3.7B).

The changes in the QRS duration following vehicle or T-2 toxin (0.5 mg/kg) administration are shown in Figure 3.8B. The QRS duration was significantly prolonged in the T-2 toxin administration group at both the first and second injection ($P<0.01$). The QRS duration was not altered by the presence of atropine (Figure 3.8E). The groups with both blockades exhibited significantly shorter QRS durations than the T-2 toxin group ($P<0.05$) (Figure 3.8E).

QT interval in short-term observation

The changes in the QT interval observed after DON-administration are shown in Table 3.3A. There were no differences in QT interval in the 0.5 mg/kg-DON or 1mg/kg-DON group compared with the control. However, in the 2.0 mg/kg-DON group, the QT interval was significantly increased at 60 min after the DON injection ($P<0.05$).

T-2 toxin with dosage of 0.5 mg/kg significantly decreased the QT interval only at 180 min following administration ($P<0.01$) as shown in Table 3.3B, while the dose of 0.1 mg/kg showed no significant changes as compared with the control.

QT interval in long-term observation and effects of autonomic nervous blockers

DON did not induce a significant change of QT duration in long-term observation (Figure 3.7B), although the tendency for dose-related increase of QT interval was observed. The QT interval in rats with the autonomic nervous blockades was not analyzed because the end of T wave in ECG was obscure in some experiments.

Changes in the QT interval observed after T-2 toxin administration are shown in Figure 3.8C. There were no significant differences in the QT interval at 0.1 mg/kg of T-2 toxin in both the first and second administrations compared with the control (Figure 3.8C). However, at the dose of 0.5 mg/kg of T-2 toxin the QT interval was significantly decreased ($P<0.05$) as compared with the control (Figure 3.8C). When compared between the first and second administrations, the QT interval was significantly prolonged at the 2nd injection ($P<0.01$). However, no significant change was recognized as compared with the control.

When atropine was pretreated, there was no significant change in any group. However, the QT interval was significantly prolonged by the pretreatment with propranolol alone ($P<0.05$) and with the combination of T-2 toxin and propranolol ($P<0.01$) when compared with the control (Figure 3.8F).

Occurrence of arrhythmia

DON administration at all doses clearly induced arrhythmia, as represented by second-degree atrioventricular (AV) block (typically following first-degree AV block), ventricular extrasystole, supraventricular extrasystole, nodal escaped beat (parasystole), and atrial bradycardia (Figure 3.9). The occurrence of arrhythmia after the DON injection is summarized in Table 3.4A. The frequency of second-degree AV block episodes increased significantly in a dose-dependent manner (ANOVA, $P<0.05$). These arrhythmia episodes usually lasted for several seconds. A relatively large number of ventricular extrasystole episodes (premature ventricular contraction) were observed without a dose-dependent manner, even at the lowest dose of DON (0.5 mg/kg, s.c.). These arrhythmia episodes appeared after 3 h, mostly at 10- 20 h, following the DON

administration. However, no obvious arrhythmias including ventricular extrasystole were observed in the control group. Moreover, DON-induced arrhythmias completely disappeared when pre-treatment with blocking agents i.e. propranolol and atropine.

T-2 toxin at 0.1 mg/kg and 0.5 mg/kg also induced second-degree AV block (typically following first-degree AV block), ventricular extrasystole, ventricular tachycardia, supraventricular extrasystole, nodal escaped beat (parasystole), and sinus bradycardia (Figure 3.10). The episodes of these arrhythmias were mostly detectable from 8 hours after toxin administration and lasted for a few days as shown in Figure 3.11. However, no obvious arrhythmias including ventricular extrasystole were observed in the control group.

The occurrence of arrhythmia during observation after T-2 toxin injection is summarized in Table 3.4B. The frequency of episodes of second-degree AV block and sinus bradycardia increased significantly in a dose-dependent manner ($P<0.01$). Each episode of arrhythmia usually lasted for several seconds. The supraventricular extrasystole appeared only in the dose of 0.5 mg/kg T-2 toxin group ($P<0.01$). A relatively large number of episodes of ventricular extrasystole (premature ventricular contraction) was observed and was mostly recognized at the lowest dose of T-2 toxin (0.1 mg/kg, s.c.). As for comparison of the first and second injections in the 0.5 mg/kg-T-2 group, a significantly higher frequency of occurrence was found for the first administration of toxin ($P<0.01$ and 0.05).

The effects of the two blockades on the occurrence of arrhythmia are shown in Figure 3.12. The second-degree AV block and sinus bradycardia significantly disappeared in both the “T-2 + atropine” and “T-2 + propranolol” groups ($P<0.01$ and $P<0.05$) (Figures 3.12A and 3.12D). Supraventricular extrasystole failed to show any

significant changes when compared with other groups (Figure 3.12B). The combination of T-2 toxin and atropine significantly increased the occurrence of ventricular extrasystole when compared with the control group ($P<0.05$) as shown in Figure 3.12C.

Heart rate variability

1) LF power in short-term observation

The changes in LF power that occurred after vehicle or DON administration are shown in Figure 3.13A. Significant differences ($P<0.05$, two-way repeated-measures ANOVA) were found for both time and doses of DON injected. Significant differences ($P<0.05$) were observed at 90 min in the 0.5 mg/kg-DON group, at 90 min and 120 min in the 1 mg/kg-DON group, and in the 2.0 mg/kg-DON group at all time-points after 90 min if compared with control group at each time point. The extent of decrease of LF power seemed to be most potent at 90 min.

T-2 toxin decreased LF power, being observed from 150 min following administration, where at the dose of 0.5 mg/kg a significant decrease in LF power was present at 150 and 180 min after the administration ($P<0.05$ and $P<0.01$, respectively). As for the lower dose, i.e., 0.1 mg/kg, a significant decrease in the LF power was observed at only 180 min following the injection when compared with the control group ($P<0.05$) as shown in Figure 3.14A.

2) LF power in long-term observation and effects of blockers

At the dose of 2 mg/kg, DON significantly decreased LF power ($P<0.01$) during one day after administration and this responses were observed in both light and dark period as shown in Figure 3.15A. As regards the lower doses of DON, i.e., 0.5 mg/kg

and 1 mg/kg, there was no significant difference in LF power during the same observation time. With the existence of blockade, the “DON + atropine” showed the significant difference from the DON-injected group in both light and dark period ($P<0.05$ and $P<0.01$, respectively), as shown in Figure 3.17A.

The changes in LF power that were observed after vehicle or T-2 toxin administration are shown in Figure 3.16A. A significant difference ($P<0.05$, two-way repeated-measures ANOVA) was observed in only the light period in the 0.5 mg/kg T-2 toxin group, with marked decreases being recognized during the 3-day observation period as compared with the control group. Moreover, there was a significant difference between the 0.5 mg/kg T-2 toxin and 0.1 mg/kg T-2 toxin groups ($P<0.01$) (Figure 3.16A). There was a marked decrease ($P<0.01$) in LF power in the atropine group without T-2 toxin (Figure 3.18A). The existence of both blockades resulted in a significant decline ($P<0.05$) in LF power as compared with the T-2 toxin group without blockades in both the light and dark periods (Figure 3.18A).

3) *HF power in short-term observation*

The changes in HF power observed after vehicle or DON administration are shown in Figure 3.13B. Significant differences ($P<0.05$, two-way repeated-measures ANOVA) were observed for both time and doses of DON injected. Significant decreases ($P<0.05$) were recognized at 90 min in the 0.5 mg/kg-DON group, at 90 min and 150 min in the 1.0 mg/kg-DON group, and at 90, 120, 150 and 180 min in 2.0 mg/kg-DON group if compared with control group at each time point.

HF power was significantly lowered than the control group when administered with 0.5 mg/kg-T-2 toxin. At dose of 0.5 mg/kg, HF power started to decrease from 90

min following administration ($P<0.05$) as represented in Figure 3.14B. From 120 to 180 min, significant differences were identified in both of 0.1 and 0.5 mg/kg-T-2 toxin ($P<0.01$) if compared with the control group.

4) HF power in long-term observation and effects of autonomic nervous blockers

DON at dose of 2 mg/kg significantly induced a decrease of HF power at only light period ($P<0.01$) as shown in Figure 3.15B. In longer observation time, no significant differences were found in the 0.5 and 1 mg/kg-DON groups in both light and dark periods. As regards the effect of blockades, a significant decrease ($P<0.01$) in HF power was recognized in "DON + atropine" group in the light period as shown in Figure 3.17B.

Changes in HF power that occurred after vehicle or T-2 toxin administration are shown in Fig. 3.16B. A significant difference ($P<0.05$, two-way repeated-measures ANOVA) similar to that found in the LF power was found in the light period in the 0.5 mg/kg T-2 toxin group compared with the control group.

A marked decrease ($P<0.01$) in HF power was recognized in the atropine group (Figure 3.18B) without T-2 toxin. In the T-2 toxin + atropine group, a significant decrease ($P<0.05$) in HF power in the light period was observed when compared with the T-2 toxin group without blockades (Figure 3.18B). There was no significant difference between the propranolol alone and T-2 + propranolol groups.

5) LF/HF ratio in short-term observation

The changes in the LF/HF ratio after vehicle or DON administration are shown in Figure 3.13C. Significant differences ($P<0.05$, two-way repeated-measures ANOVA) were found for both time and doses of DON injected. At 90 min after DON injection, all DON groups showed a significant decrease ($P<0.05$) in the LF/HF ratio compared with that in the control group. This significant decrease was also recognized at 120 min in the 2.0 mg/kg-DON group ($P<0.05$).

T-2 toxin at dose of 0.1 mg/kg, induced a significant increase in LF/HF ratio at 30 ($P<0.05$), 150 ($P<0.01$) and 180 min ($P<0.01$) following administration as represented in Figure 3.14C. On the contrary, in the same dose at 60 min after the injection, the LF/HF ratio was significantly decreased if compared with the control ($p<0.01$). No significant differences were observed in the 0.5 mg/kg-T-2 toxin administered group.

6) LF/HF ratio in long-term observation and effects of autonomic nervous blockers

The alteration of LF/HF ratio in longer time of observation following DON-injection is represented in Figure 3.15C. The significant differences were identified in the light period of 0.5 and 1 mg/kg-DON administration ($P<0.05$) if compared with the control. DON at dose of 2 mg/kg failed to show any significant change from the control group. With the atropine infusion, the LF/HF ratio is significantly lower than that in DON-administration group without atropine, which was observed only in the light period ($P<0.01$) as shown in Figure 3.17C. In the similar way, the “DON + propranolol” group showed a significant decrease ($P<0.01$) of LF/HF ratio in the dark period if compared with the DON-administration group and was observed (Figure 3.17C).

The changes in the LF/HF ratio after vehicle or T-2 toxin administration are shown in Figure 3.16C. No significant differences were identified in this parameter. However, there was a contrasting response between the 0.1 and 0.5 mg/kg T-2 toxin groups. In the dark period in the 0.1 mg/kg T-2 toxin group showed an increased trend in LF/HF ratio compared with the control group, while the 0.5 mg/kg T-2 toxin group showed a decreased trend in both the light and dark periods.

With the blockades, the LF/HF tended to decrease in every group except in the dark period in the atropine group, which showed a significant increase as compared with the control ($P<0.05$) as shown in Figure 3.18C. Both the "T-2 toxin + atropine" and "T-2 toxin + propranolol" groups had significant decreases in the LF/HF ratio when compared with the T-2 toxin administration without blockades ($P<0.05$ and 0.01, respectively) (Figure 3.18C).

7) Total power in short-term observation

The changes in total power that occurred after vehicle or DON administration are shown in Figure 3.13D. Significant differences ($P<0.05$, two-way repeated-measures ANOVA) were found for both time and doses of DON injected. The total power was significantly increased at 30 min after the DON injection in all DON groups, while significant decrease ($P<0.05$) was recognized from 90 to 150 min in 1.0 mg/kg- and 2.0 mg/kg-DON groups and also at 180 min 2.0 mg/kg-DON group compared with that in the control.

T-2 toxin significantly decreased total power, which was found after 120 min following administration. At 120 min, 0.5 mg/kg-T-2 toxin showed a significant decrease if compare with the control ($P<0.01$) as shown in Figure 3.14D. The total

power was also significantly lowered than the control from 150 to 180 min following 0.5 mg/kg-T-2 toxin administration ($P<0.01$) (Figure 3.14D).

8) Total power in long-term observation and effects of autonomic nervous blockers

At doses of 1 and 2 mg/kg, DON administration significantly reduced total power as represented in Figure 3.15D ($P<0.01$). However, this reduction was observed only in the dark period, and no significant difference was observed in 0.5 mg/kg-DON injection. With the existence of blockades, the "DON + atropine" group showed a significant decrease ($P<0.01$) in the light period if compared with the DON-injection group without atropine, as shown in Figure 3.17D. On the contrary, the "DON + propranolol" significantly increased the total power in both light and dark period if compared with the DON-administration group without propranolol ($P<0.01$) (Figure 3.17D).

The changes in total power that occurred after vehicle or T-2 toxin administration are shown in Figure 3.16D. A significant difference ($P<0.01$, two-way repeated-measures ANOVA) was found in the 0.5 mg/kg T-2 toxin group, but not in 0.1 mg/kg T-2 toxin group where the decrease in total power was recognized during the light period ($P<0.01$) compared with the control for 3-day observation period.

The total power significantly increased in the propranolol group in the dark period and further increased in the "T-2 toxin+propranolol" group as shown in Figure 3.18D. In both the light and dark periods, "T-2 toxin + propranolol" produced a significant increase in total power when compared with T-2 toxin administration alone ($P<0.01$).

Discussion

It has been described by several studies with acute experiments using anesthetized laboratory animals or *in vitro* experiments that T-2 toxin has some cardiovascular toxicities; the majority of these studies were conducted in the 1980s (Schoental et al., 1979; Sherman et al., 1986; Borison and Goodheart, 1989). However, the toxicity of DON on the cardiovascular system has not been widely identified yet. The present study was performed to elucidate the properties of cardiac toxicity due to representative trichothecene mycotoxins in conscious and unrestrained rats using recent techniques involving a telemetric measurement and power spectrum analysis of HRV, especially focusing on the appearance of arrhythmias, changes in ECG components, and alterations in the autonomic nervous activity.

The present study elucidated that the administration of DON and T-2 toxin induced significant cardiac toxicities. The observations in this chapter were performed at least within 3 h and utmost for 3 days. According to the result, it is clarified that DON has less toxic effect, as the toxicity was shown to be decayed within 6 h, whereas T-2 toxin has the stronger effects and responses induced by T-2 toxin administration lasted for as long as 72 h following the subcutaneous administration.

Within 3 h of observation, both mycotoxins induced an increased heart rate with changes in ECG-wave forms. Many of the components involved in heart rate regulation in normal rats are largely influenced by the balance of autonomic nervous activity. Therefore, the increased heart rate that was observed in the toxin treatment groups in the present study might have been due to either an increase in the sympathetic nervous activity or a decrease in the parasympathetic nervous activity. In a earlier study, dogs

subjected to intravenous injection with T-2 toxin (2.0 mg/kg) displayed an increased heart rate within 45±15 minutes of the injection that lasted for at least 4 h and were accompanied by decreased arterial blood pressure (92-83 mmHg), while such changes in heart rate were inhibited by pretreatment with propranolol (Bubien and Woods, 1987). This study also revealed mild prolongation of cycle length, i.e., slowing of heart rhythm, by recording action potentials from canine sinus node cells. In the present study, the HF power, an index of the parasympathetic nervous activity, was decreased following the DON and T-2 toxin injections. However, the LF power and LF/HF ratio were significantly decreased after the DON and T-2 toxin-injection groups. Such results from the power spectrum analysis of LF components were not consistent with the increased heart rate observed after the DON and T-2 toxin-injection. Thus, the increase in heart rate observed after the DON and T-2 toxin administration in this study might have been partly derived from the direct toxicity of DON and T-2 toxin to the heart in addition to the autonomic nervous effects on the heart via the attenuation of the HF component. This marked alteration of the power spectrum resulted in a potent decrease in total power in the 2.0 mg/kg- and 1.0 mg/kg-DON groups and in the 0.1 mg/kg- and 0.5 mg/kg-T-2 toxin groups. This suggests that the entire outflow of the autonomic nervous system was inhibited in the rats with administration of high doses of DON and T-2 toxin .

The minor extent of prolongation of PR interval and QT interval was observed at 60 min and 120 min after 2.0 mg/kg-DON injections. The PR prolongation also appeared at 90 min following 0.5 mg/kg-T-2 toxin administration. However, QRS duration and QT interval become shorten within 3 h of observation. These might be derived from the shortening of R-R interval due to the increase of HR. Those changes

may reflect weak toxic effects of these trichothecene mycotoxins on the atrioventricular conduction system and cardiac muscles immediately after the administration with high dose of DON and T-2 toxin. It has recently been reported that high concentrations of DON (50, 100, or 200 mg/L) induced decreases in action potential parameters including a prolongation of the action potential duration (APD) and the maximum rate of depolarization (Vmax) in cultured cardiomyocytes isolated from neonatal rats (Peng and Yang, 2003). If such effects are exhibited in myocytes, it is likely that the trichothecene mycotoxin, such as DON and T-2 toxin, modifies cardiac ion channels involved in the depolarization and/or repolarization in which the effects might depend on the concentration of DON and T-2 toxin administered.

As for longer observation, DON almost lost its toxicity within a day of injection while T-2 toxin induced significant cardiac toxicities, that is, the frequent appearance of arrhythmia accompanied by an increased heart rate and the prolongation of QRS duration and QT interval during three days after the toxin administration. Sirén & Feuerstein (1986) reported that in conscious rats measurement by a Doppler technique revealed a potent increase in vascular resistance and gradual decrease in blood flow during 6 hours after intravenous injection of T-2 toxin at a dose of 1 mg/kg. These findings suggested that T-2 toxin could induce changes in blood pressure with hypertension or hypotension accompanied by a decrease or increase in HR after the administration of this toxin. Therefore, it should be considered that different cardiovascular alterations depending on the time course and animal species might be present in response to T-2 toxin. If hypertension due to increased vascular resistance is caused immediately after the administration of T-2 toxin, a decrease in heart rate could be elicited, and if hypotension, which may follow the initial hypertension, is caused, an

increase in heart rate could be induced by the baroreflex mechanism. This suggests that the increased heart rate observed in the 2 mg/kg DON and 0.5 mg/kg T-2 toxin group in the present study was due to sympathetic nervous activity since significant inhibition of the heart rate was produced by the preceding and continuous administration of propranolol. Although the extent of decrease in heart rate by propranolol was below the control level without DON and T-2 toxin, especially in the dark period, it might be suggested from the result of the sympathetic blockade that the increased heart rate during the observation time (one day for DON; three days for T-2 toxin) after administration did not seem to have originated from direct actions of trichothecene mycotoxin on the sinoatrial node.

The LF and HF power were significantly decreased by the administration of 2 mg/kg of DON and 0.5 mg/kg of T-2 toxin. The ratio of LF/HF power, an index of balance in the autonomic nervous activity, also significantly decreased at the same time. These changes in the HRV analysis did not indicate any predominant sympathetic nervous activity, although there was a trend of increase in LF/HF ratio at the lower dose of toxin (1 mg/kg of DON and 0.1 mg/kg of T-2 toxin). Therefore, there was a discrepancy between the increase in heart rate and the results of HRV analysis at the dose of 2 mg/kg-DON and 0.5 mg/kg-T-2 toxin. This discrepancy might be partly due to the decrease in overall power at higher dose of trichothecene mycotoxin. In some conditions associated with sympathetic activation, the resulting tachycardia is usually accompanied by a marked reduction in total power, whereas the reverse occurs during vagal activation (Pomeranz et al., 1985). The marked alteration of the power spectrum suggests that the entire outflow of the autonomic nervous system was inhibited in rats administered a high dose of trichothecene mycotoxin. In the present study, the decrease

in total power was remarkable in the light period. It is considered that the decreasing effect of the toxin on total power might be easier to observe in the light period than in dark period because the parasympathetic nervous tone, which produces increases in HF and total powers, is basically high in the light period compared with the dark period. On the other hand, at the dose of 0.1 mg/kg-T-2 toxin, the LF/HF ratio was high in the dark period, suggesting that the autonomic nervous activity in rats with low concentrations of T-2 toxin is not largely influenced or somewhat tends to be sympathetic predominant.

A minor extent of prolongation and shortening of PR interval at 0.1 mg/kg and 0.5 mg/kg of T-2 toxin was observed for the second injection of the toxin while there were no any significant changes of ECG-waveform observed within a day following DON administration. This indicates that DON-induced abnormalities are reversible when the toxin is completely eliminated from the body, whereas T-2 toxin's toxicity seems to be irreversible since significant changes still could be identified during three day of observation. However, this might be derived from a residual of T-2 toxin that would be completely eliminated at 72 h in addition to some possible tissue damages at early stage. The significant prolongation of QRS duration was observed at 0.1 mg/kg and 0.5 mg/kg of T-2 toxin. This change in QRS duration may be partly derived from direct toxic actions of T-2 toxin on the cardiac muscle since Bubien & Woods (1986) reported decreased resting potentials and action potential durations in the cardiac muscle cell in an experiment with recordings of canine heart tissues. However, in the present study, the significant shortening effect on QRS duration by pretreatment with both blockades indicated that the elongation of QRS duration by T-2 toxin alone was influenced by the autonomic nervous function. In general, it has been believed that the ventricular muscle is innervated by the sympathetic nerve but not by the

parasympathetic nerve. A possibility that the shortening of QRS duration by atropine was associated with an increase in heart rate (shortening of the RR interval) was suggested. However, the QRS duration was also shortened by propranolol administration despite the heart rate being decreased by this drug. The shortening of QRS duration by propranolol was inconsistent with the general concept about the positive correlation between the QRS duration and RR interval within a normal range for heart rate. Mild intoxication due to continuous administration of the beta-blocker may be a possible explanation for this discrepancy since intoxication involves significant shortening of QRS duration as well as QT interval in humans (Love J. N. et al., 2002).

In the present study, no obvious arrhythmias such as second-degree AV block, ventricular extrasystole, or supraventricular extrasystole were observed immediately after DON administration. Instead, they appeared after 3 hrs, mostly at 10-20 hr after DON administration. This finding indicates that DON administration takes time to produce an arrhythmogenic condition in the heart of rats. A previous study in which the protein levels in the heart, liver, kidneys, and spleen were examined in mice that had been intraperitoneally injected with DON and sacrificed 5 hrs later showed that DON with a dose of 20 or 80 mg/kg caused metabolic damage to myocytes by inhibiting protein synthesis in mice (Robbana-Barnat et al., 1987). This study also demonstrated the histological changes represented by pericardial calcifications observed in most mice after 15 or 21 days of 20 ppm -DON ingestion.

In some studies, mitogen-activated protein kinases were suggested to play a role in the expression of DON-induced apoptosis (Baltriukiene, et al., 2007; Pestka, 2008). It is feasible to consider that the occurrence of arrhythmias, such as the premature

ventricular contraction observed during the period from 10-20 hr after the DON administration, is, at least in part, associated with the cardiac toxicity caused by intracellular metabolic disorders in myocytes. Furthermore, it would be interesting to examine whether DON can induce apoptosis by producing oxidative stress in cells. A recent study was suggested that the DNA damage was induced by direct application of DON to the human hepatoma cell line (HepG2) cells and that the DNA damage was caused by oxidative stress (Zhang, 2009). Although it was not elucidated whether the DON administration in our study induced oxidative stress in myocytes, cardiac lesions caused by such mechanisms might be considered as a causal factor of the arrhythmia, especially ventricular tachycardia, observed in this study.

In the present study, with the exception of transient exhibition of sinus bradycardia, no obvious arrhythmias such as second-degree AV block, ventricular extrasystole, or supraventricular extrasystole were observed immediately after T-2 toxin administration. Instead, they predominantly appeared after 8 hours, and the arrhythmias lasted for the three days of observation after the T-2 toxin administration. This finding indicates that T-2 toxin administration takes time to produce the arrhythmogenic condition and that the cardiac influence lasted for at least three days. Although it is feasible to consider that direct toxic effects (Bunner & Morri, 1988) on myocytes were associated with the occurrence of arrhythmia, most of the second-degree AV blocks and sinus bradycardia in the present study seemed to be induced or augmented by the vagal activity since such a conduction disturbance was largely diminished by the atropine treatment.

However, according to the results of Chapter 2, arrhythmias were identified during 3 h of observation. A difference in the time of arrhythmic occurrence might be derived from the rat's condition as in Chapter 2 ECG-waveform was recorded under anesthetic condition but in this chapter all the parameters were recorded under conscious condition. Zorniak et al., (2010) has reported that the use of urethane for anesthetic induction could affect hemodynamic parameters such as HR, blood pressure and index myocardial oxygen consumption. It is feasible that the arrhythmias in Chapter 2 might receive the effect from anesthetic agent, i.e., urethane that was used in the present study.

If T-2 toxin induced hypotension for a few days following administration of T-2 toxin, it should provoke a reflex inhibition of the vagal activity and augmentation of the sympathetic nerve activity. From the results of an acute experiment in the dog, the reduction in arterial blood pressure and increase in heart rate were suggested to be mediated by the sympathetic nervous system (Bubien & Woods, 1987). In the present study, it was assumed that the tonus of the parasympathetic nervous activity was enhanced intermittently to regulate heart rate and systemic blood pressure by means of antagonistic neural mechanisms during basically high tension of the sympathetic nervous activity for a longer period after the administration of T-2 toxin. Such events of parasympathetic activation might cause a momentary decrease in firing rate at the sinoatrial node and also produce second-degree AV blocks, which lead to a slowing of atrioventricular conduction.

The casual relationship between arrhythmias and the decrease in LF, HF and total powers observed at 0.5 mg/kg of T-2 toxin in the present study was unclear. Although the arrhythmias such as sinus bradycardia and atrioventricular blocks are considered to

be associated with momentary alteration of balance between the sympathetic and parasympathetic nervous activities, such nervous activities may be not necessarily reflected the HRV analysis.

A significant increase in occurrence of ventricular extrasystoles was identified with the combination of 0.5 mg/kg-T-2 toxin and atropine. The atropine enhanced oxygen consumption as a result of tachycardia (Westenskow et al., 1981), which results in an increase in workload of the heart and produces cardiac conditions tending to cause ventricular extrasystoles. It is known that tachycardia produces an accumulation of superoxide anion released from the mitochondrial electron transport chain in myocytes (Han et al., 2001). In addition, T-2 toxin itself has been clarified to be a substance producing oxidative stress in tissues involving the brain (Chaudhari et al., 2009, 2010; Doi & Uetsuka, 2011). Although the detail mechanism of ventricular extrasystoles observed in the present study was not elucidated, the oxidative stress, to some extent, may be attributed to this arrhythmia, as it has been reported that the number of cases of ventricular extrasystole in cases of myocardial reperfusion was significantly decreased by treatment with superoxide dismutase (Kónya et al., 1992).

In conclusion, the results of the present study demonstrated that DON is also toxic to the heart at doses of 0.5 mg/kg S.C. and higher, disturbing the cardiac conduction and excitation system as well as T-2 toxin which has a property of potent cardiac toxicity; the appearance of the toxicity was largely influenced by the autonomic nervous activity, lasting for one day in DON and for at least three days in T-2 toxin in conscious rats.

2.5 Summary

The longer responses in cardiac and autonomic nervous functions were investigated in unanesthetized and unrestrained rats that were implanted with a telemetry transmitter and with administration of DON and T-2 toxin. Both toxins exhibited arrhythmia, involving conduction disturbances and ventricular extrasystoles, and changes in ECG wave components, being accompanied by alterations in the autonomic nervous function. Such cardiac abnormalities were less in DON compared to T-2 toxin, where most of toxic effects of DON disappeared within 12 h and the effects of T-2 toxin lasted for as long as 72 h following the subcutaneous administration. Besides these findings, the possibility of direct actions of these toxins to the cardiac muscle cannot be rejected since the ventricular arrhythmia still appeared even if the autonomic nervous regulation to the heart was blocked by blockades, i.e., atropine and propranolol.

Table 3.1

A. PR intervals in the control and DON-groups

Time (min) after administration with vehicle (control) or DON (0.5-2 mg/kg)							
	0	30	60	90	120	150	180
Control	42.17±1.74	42.71±2.37	43.36±2.96	43.77±2.94	42.73±2.59	44.21±2.64	43.12±2.52
0.5 mg/kg	43.83±3.32	43.07±3.50	43.53±2.00	44.03±1.96	44.17±2.47	43.33±4.98	44.50±3.19
1 mg/kg	41.40±0.75	44.72±6.65	43.97±2.53	44.37±1.44	43.88±2.29	43.13±2.21	44.48±2.97
2 mg/kg	40.17±5.65	47.08±8.32	46.06±4.62	49.29±4.86*	44.96±2.50	43.38±2.78	45.24±3.72

*: Significantly different from the control value (p<0.05). DON: Deoxynivalenol. Control: the control group administered the vehicle injection. Each value is expressed as the mean ± SEM. Note that a significant change in the PR interval was observed at 90 min after the administration of 2 mg/kg-DON.

B. PR intervals in the control and T-2 toxin-groups

Time (min) after administration with vehicle (control) or T-2 toxin (0.1, 0.5 mg/kg)							
	0	30	60	90	120	150	180
Control	45.33±8.73	43.75±5.78	36.13±4.18	42.25±4.78	41.75±3.47	42.00±3.15	57.13±7.41
0.1 mg/kg	54.60±5.85	35.75±5.53	43.00±9.83	43.75±5.63	45.25±7.41	41.25±4.97	48.50±6.76*
0.5 mg/kg	34.25±8.23	38.75±10.05	36.50±8.86	54.50±8.06**	47.75±2.66	49.25±1.80*	46.00±6.99**

**, *: Significantly different from the control value (p<0.01, p<0.05, respectively). Control: the control group administered the vehicle injection. Each value is expressed as the mean ± SEM. Note that a significant change in the PR interval was observed at 90, 150 and 180 min after the administration of 0.1 or 0.5 mg/kg-T-2 toxin.

Table 3.2

A. QRS duration in the control and DON-groups

	Time (min) after administration with vehicle (control) or DON (0.5-2 mg/kg)						
	0	30	60	90	120	150	180
Control	17.50±2.95	16.36+3.59	15.09+2.59	15.58+4.25	16.09+4.95	16.64+5.17	15.91+3.70
0.5 mg/kg	18.33±4.14	17.07+4.84	14.40+2.48	15.00+2.07	15.00+2.21	16.23+2.95	15.50+1.70
1 mg/kg	19.00±2.45	16.73+2.88	15.67+3.12	14.70+3.20	17.00+2.02	16.07+2.52	15.63+2.20
2 mg/kg	14.00±2.31	19.17+4.27	15.75+3.89	18.04+5.42	18.46+5.65	16.92+5.05	17.31+3.71

DON: Deoxynivalenol. Each value is expressed as the mean ± SEM. Control: the control group administered the vehicle injection. Note that no group showed a significant difference from the control.

B. QRS duration in the control and T-2 toxin-groups

	Time (min) after administration with vehicle (control) or T-2 toxin (0.1, 0.5 mg/kg)						
	0	30	60	90	120	150	180
Control	25.67±4.61	25.88+2.94	24.00+5.39	21.25+3.23	21.63+2.78	22.75+1.67	20.50+1.59
0.1 mg/kg	21.00±3.48	25.50+3.40	24.25+3.62	20.50+1.85	20.00+2.38	18.25+0.85*	22.00+3.49
0.5 mg/kg	31.25±4.31	21.25+1.11*	21.75+2.50	18.50+0.65	19.50+1.04	23.00+3.58	21.25+3.22

*: Significantly different from the control value ($p<0.05$). Control: the control group administered the vehicle injection. Each value is expressed as the mean ± SEM. Note that a significant change in the QRS duration was observed at 30 and 150 min after the administration of 0.5 and 0.1 mg/kg-T-2 toxin, respectively.

Table 3.3

A. QT interval in the control and DON-groups

	Time (min) after administration with vehicle (control) or DON (0.5-2 mg/kg)						
	0	30	60	90	120	150	180
Control	68.33±3.92	69.85+2.11	69.62+2.04	71.98+2.88	73.71+2.67	71.92+2.57	71.57+2.95
0.5 mg/kg	68.67±3.02	67.78+1.93	70.33+1.89	69.72+2.98	69.44+2.37	72.33+3.54	65.58+2.98
1 mg/kg	65.80±3.12	69.17+2.03	71.78+1.52	68.67+3.62	70.28+2.79	66.28+3.97	67.29+3.24
2 mg/kg	72.50±4.84	72.31+4.61	80.67+4.17*	78.28+6.28	78.17+4.06	71.94+3.17	72.83+3.59

DON: Deoxynivalenol. Control: the control group administered the vehicle injection. *: Significantly different from the control value (p<0.05). Each value is expressed as the mean ± SEM. Note that a significant change in the QT interval was observed at 60 min after the administration of 2 mg/kg-DON.

B. QT interval in the control and T-2 toxin-groups

	Time (min) after administration with vehicle (control) or T-2 toxin (0.1, 0.5 mg/kg)						
	0	30	60	90	120	150	180
Control	71.17±2.10	71.88±1.61	75.13±6.03	81.00±4.02	78.50±4.54	81.88±3.63	81.13±3.80
0.1 mg/kg	76.40±3.25	70.25±6.09	74.50±3.23	82.25±2.84	79.75±6.65	86.75±1.70	84.00±4.10
0.5 mg/kg	70.75±2.87	75.00±6.10	71.75±6.93	80.25±6.57	74.50±8.67	78.00±10.50	68.75±7.50**

Control: the control group administered the vehicle injection. **: Significantly different from the control value (p<0.01). Each value is expressed as the mean ± SEM. Note that a significant change in the QT interval was observed at 180 min after the administration of 0.5 mg/kg-T-2 toxin.

Table 3.4

A. Occurrence of arrhythmias in the control and DON-groups

Arrhythmia	Dose (mg/kg)			
	0	0.5	1	2
Second-degree AV block*	0/6(0)	2/6(0.5)	4/6(1.2)	6/6(2.33)
Supraventricular extrasystole	0/6(0)	3/6(0.83)	2/6(0.6)	3/6(2)
Ventricular extrasystole	0/6(0)	4/6(1.17)	5/6(1.6)	5/6(2)

A significant dose-dependent increase was observed for second-degree AV block (*p<0.05). No evidence of these arrhythmias appeared in the 0-mg/kg DON group (vehicle). In the data, the occurrence of arrhythmia was calculated as an amount of animal in which the representative arrhythmia was identified (n=6/group). The average of occurrence of arrhthymia in each rat was represented in the parentheses. DON: Deoxynivalenol.

B. Occurrence of arrhythmias in the T-2 toxin-groups

Type	Dose (mg/kg)				
	vehicle (n=11)	0.1 (n=6)		0.5 (n=5)	
		1st inj.	2nd inj.	1st inj.	2nd inj.
Second-degree AV block	2.09±0.81	2.83±1.01	1.67±1.28	7.8±2.56**	4.2±1.24#
Supraventricular extrasystole	0.55±0.28	-	-	3.8±2.58**	2.2±1.71#
Ventricular extrasystole	0.09±0.09	1.83±0.48**	2.67±1.43**	1±0.45	-
Atrial bradycardia	0.27±0.14	1±0.37*	0.67±0.49	2.4±1.25**	1.2±0.73##

The mean frequency of arrhythmias in each rat are analyzed for 3 days interval and expressed as means ± SEM. Significant differences (*p<0.05, **p<0.01) from the control before T-2 toxin injection. Significant differences (*p<0.05, ##p<0.01) from values of the 1st injection. -, No corresponding arrhythmia found.

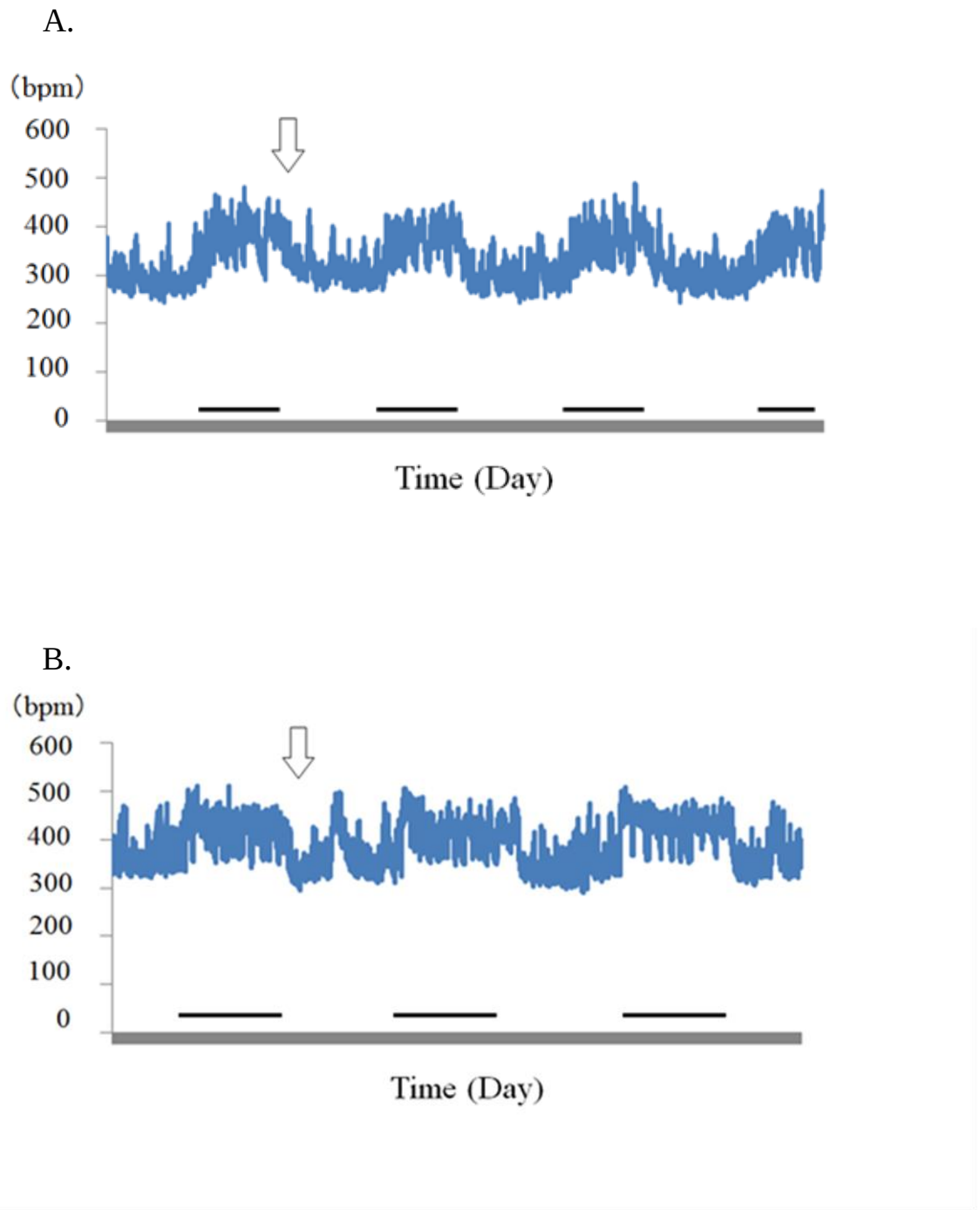


Figure 3.1 Representative changes in heart rate by recording telemetric ECG in rats The vehicle solution in A (upper panel; control group) and 1.0 mg/kg of DON in B (lower panel; DON-group) were subcutaneously injected at the point indicated by the arrows. The underlined section represents the dark period.

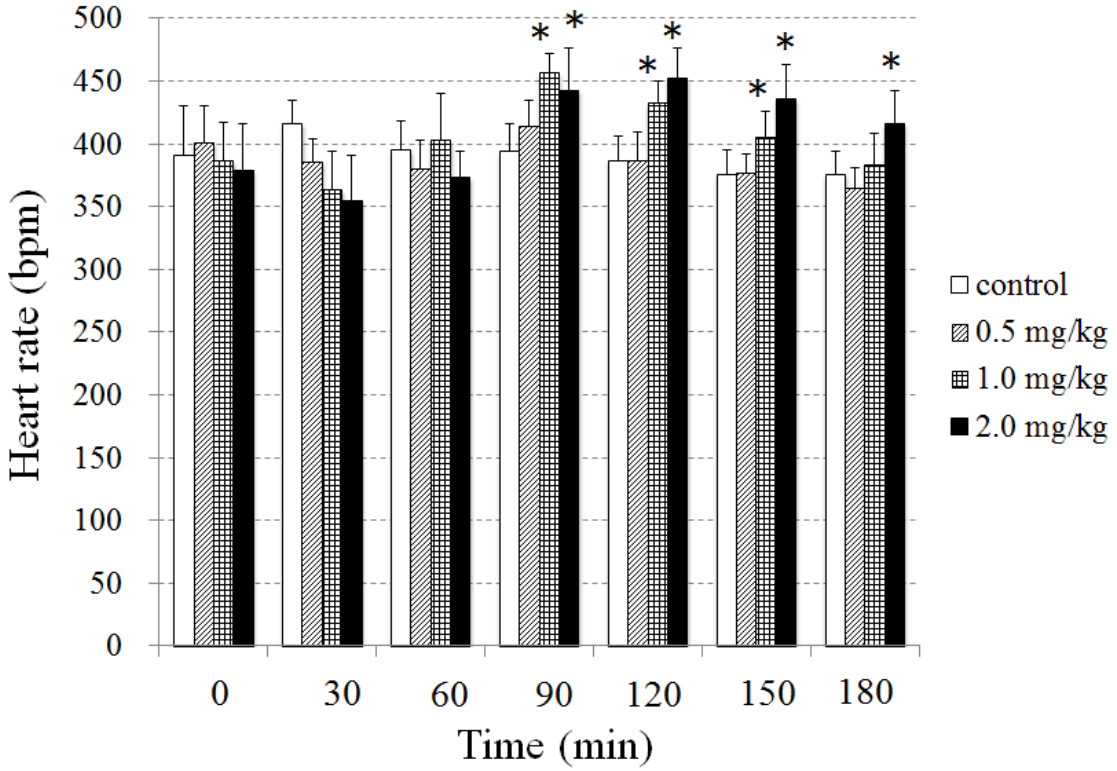


Figure 3.2 Short-term changes in heart rate in response to DON-injection
The asterisks show significant differences at $p<0.05$ (*) from the control value at each time point. Zero min is the time before the vehicle- or DON-injection. Data are expressed as means ± SEM.

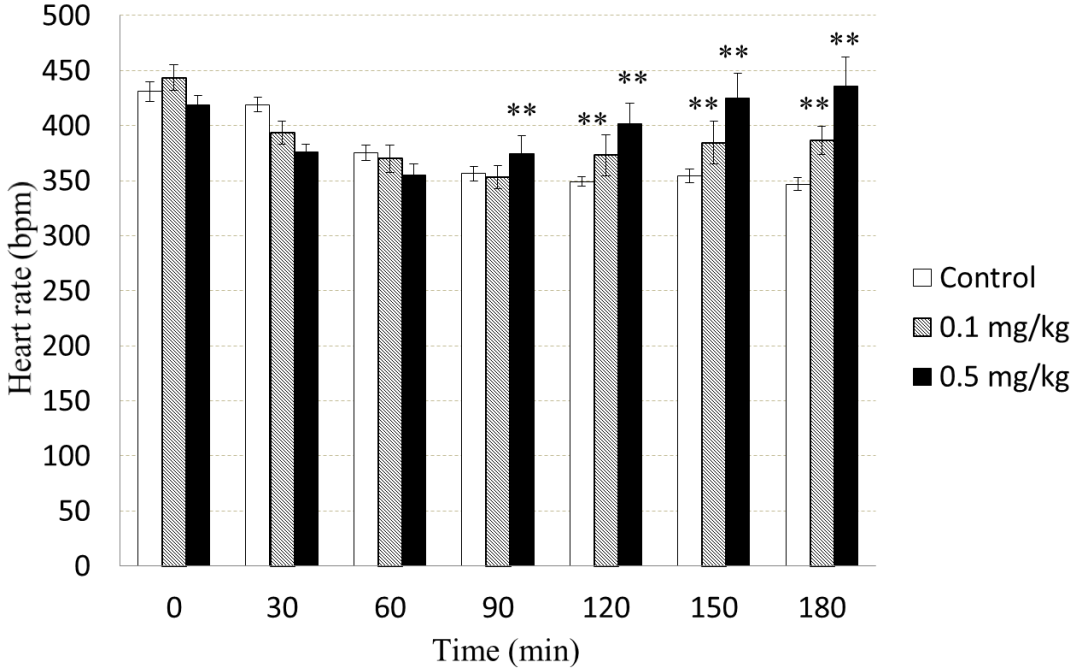


Figure 3.3 Short-term changes in heart rate in response to T-2 toxin-injection
The asterisks show significant differences at $p<0.01$ (**) from the control value at each time point. Zero min is the time before the vehicle- or T-2 toxin-injection. Data are expressed as means ± SEM.

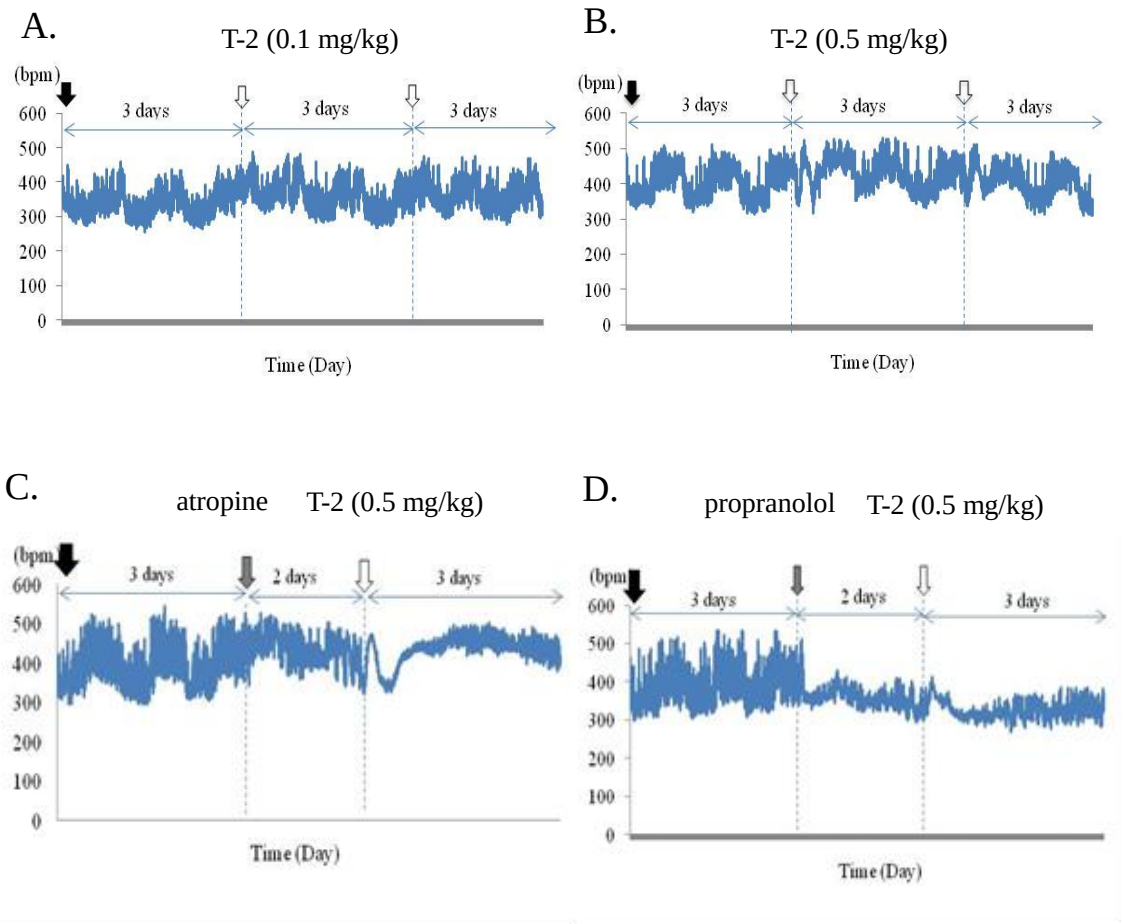
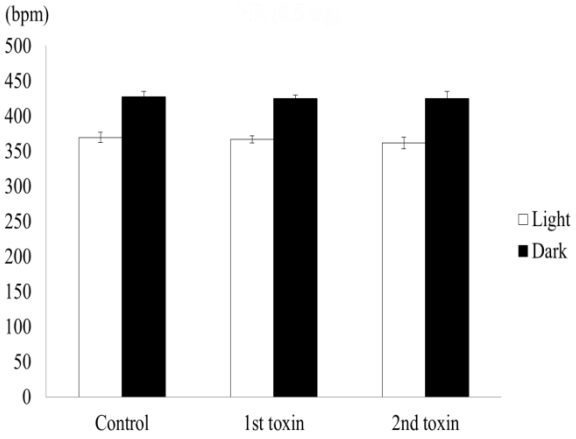
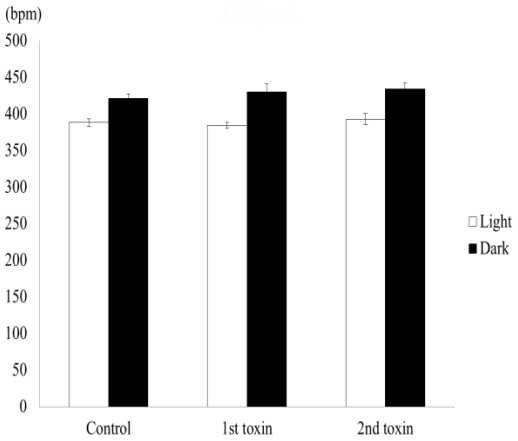


Figure 3.4 Representative changes in heart rate by recording telemetric ECG in rats. A, 0.1 mg/kg-T-2 toxin; B. 0.5 mg/kg-T-2 toxin; C. 0.5 mg/kg-T-2 toxin + atropine (20 mg/kg/day) ; D, 0.5 mg/kg-T-2 toxin + propranolol (100 mg/kg/day). The arrows indicate administration of vehicle (solid black arrow), T-2 toxin (blank arrow) and intraperitoneal implantation of mini-pump with blockades (shadow grey arrow).

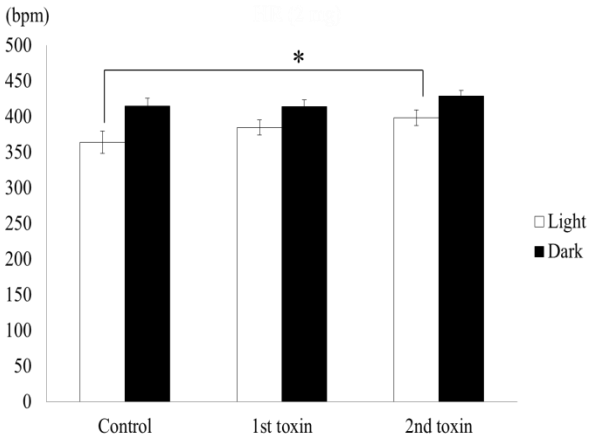
A.


(bpm)
500
450
400
350
300
250
200
150
100
50
0
Control
1st toxin
2nd toxin
Light
Dark

B.


(bpm)
500
450
400
350
300
250
200
150
100
50
0
Control
1st toxin
2nd toxin
Light
Dark

C.


(bpm)
500
450
400
350
300
250
200
150
100
50
0
*
Control
1st toxin
2nd toxin
Light
Dark

D.

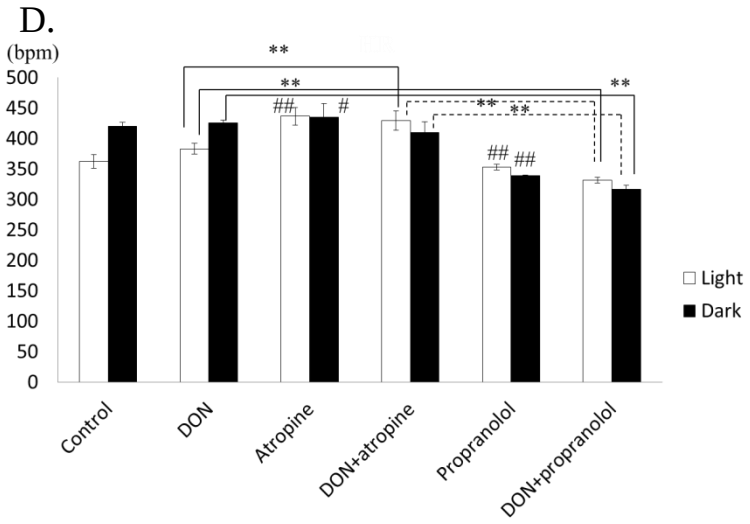

(bpm)
500
450
400
350
300
250
200
150
100
50
0
**
**
**
**
**
##
#
##
##
Control
DON
Atropine
DON+atropine
Propranolol
DON+propranolol
Light
Dark

Figure 3.5. Changes in heart rate in response to DON-injection.
A: 0.5 mg/kg of DON (n=6), B: 1 mg/kg of DON (n=6), C: 2 mg/kg of DON (n=6), D: A comparison among control (vehicle), DON (2 mg/kg), blockades, and DON (2 mg/kg) with blockades (n=5 in each condition). **: significant difference (p<0.01) between corresponding bars. Number signs: significant difference (#p<0.05, ##p<0.01) from the control. Data were based on mean values obtained for each 1 day and expressed as means ± SEM.

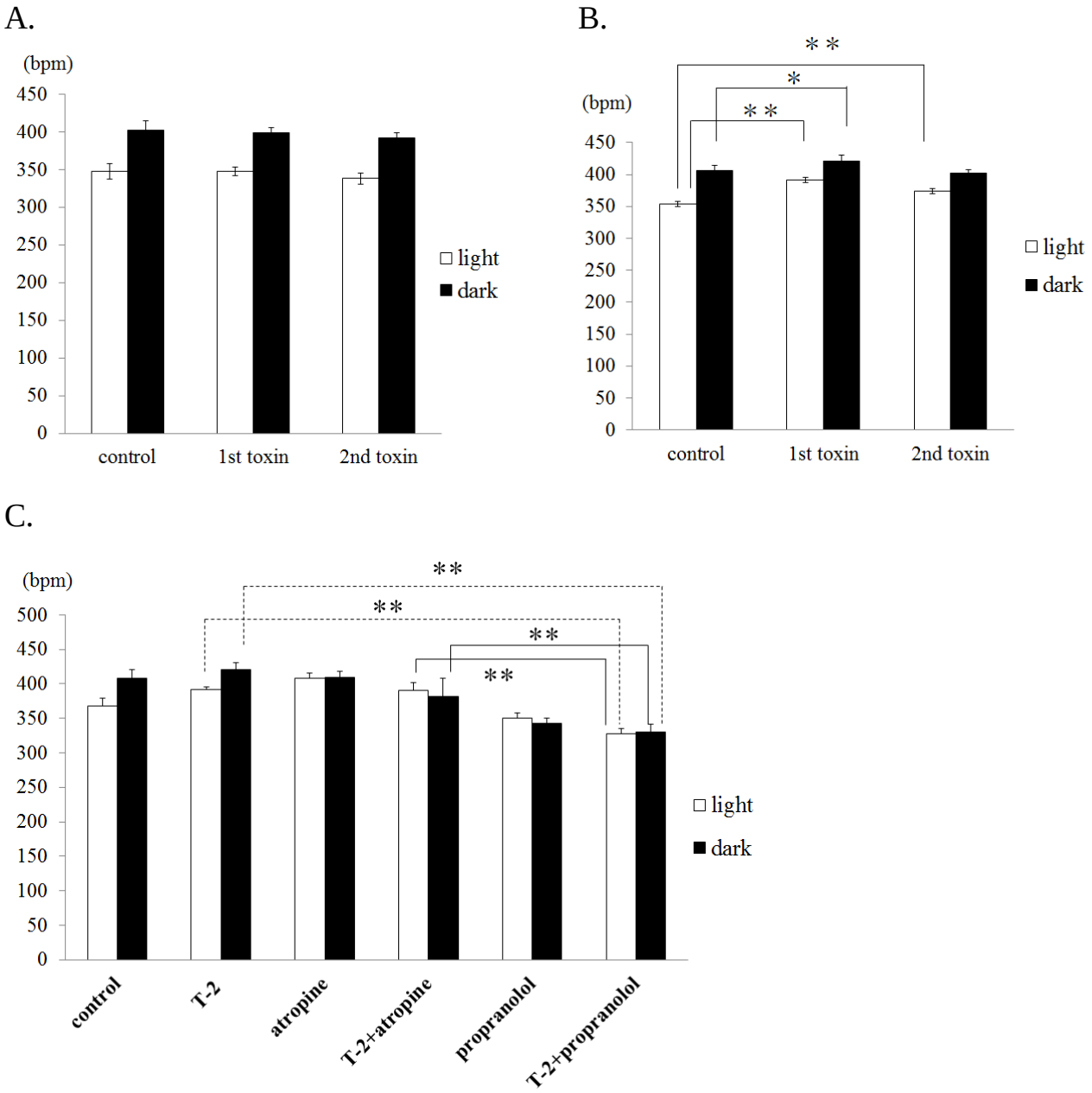


Figure 3.6. Changes in heart rate in response to T-2-injection.

A: 0.1 mg/kg of T-2 toxin (n=6), B: 0.5 mg/kg of T-2 toxin (n=5), C: A comparison among control (vehicle), D: T-2 toxin (0.5 mg/kg), blockades, and T-2 toxin (0.5 mg/kg) with blockades (n=5 in each condition). *,**: significant differences (*p<0.05,**p<0.01) between corresponding bars. Data were based on mean values obtained for each 3 days and expressed as means ± SEM.

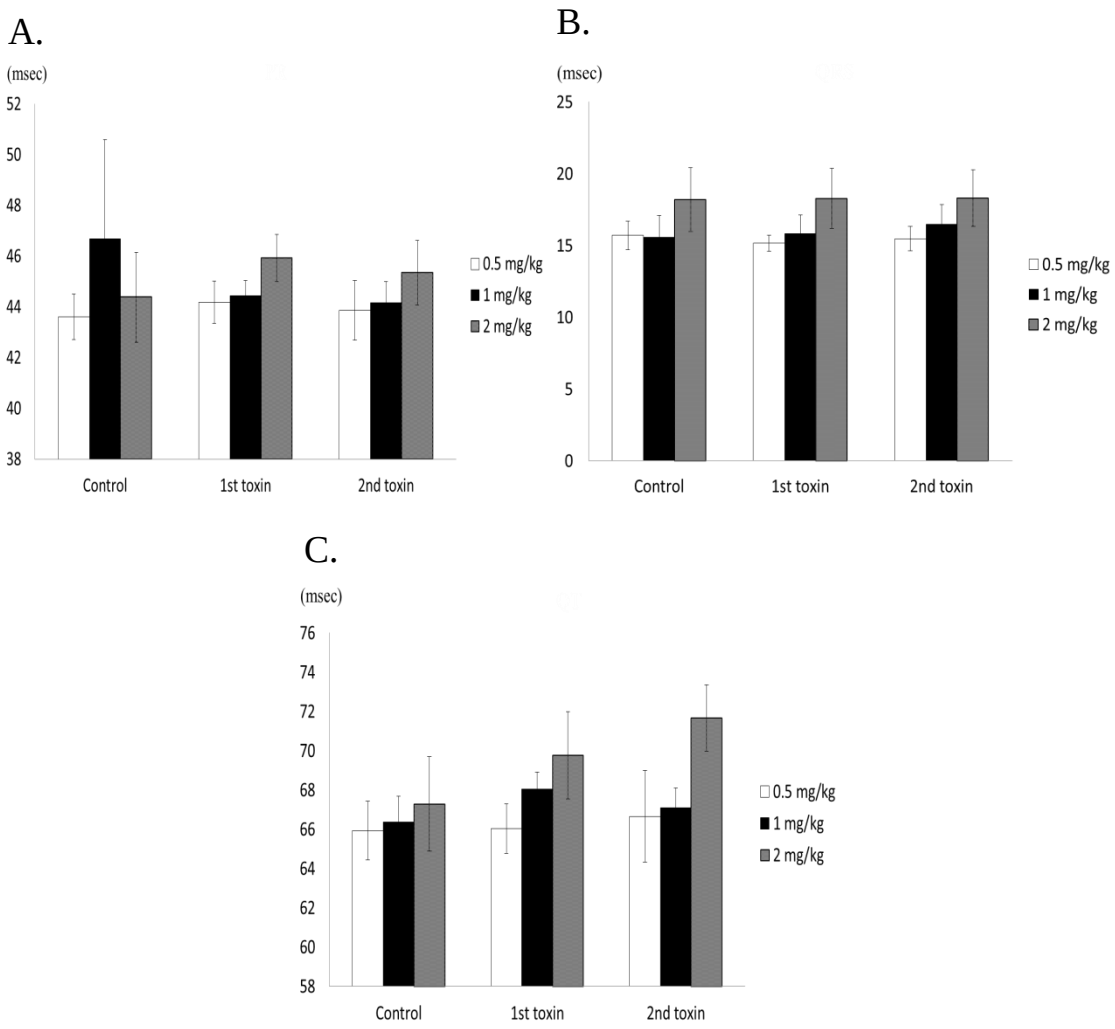


Figure 3.7. Changes in interval components of ECG in response to DON-injection.
A: PR-interval, B: QRS duration, C: QT-interval.
(No significant differences were identified when compared with the control.)
Data were based on mean values obtained for each 3 days and expressed as means ± SEM.

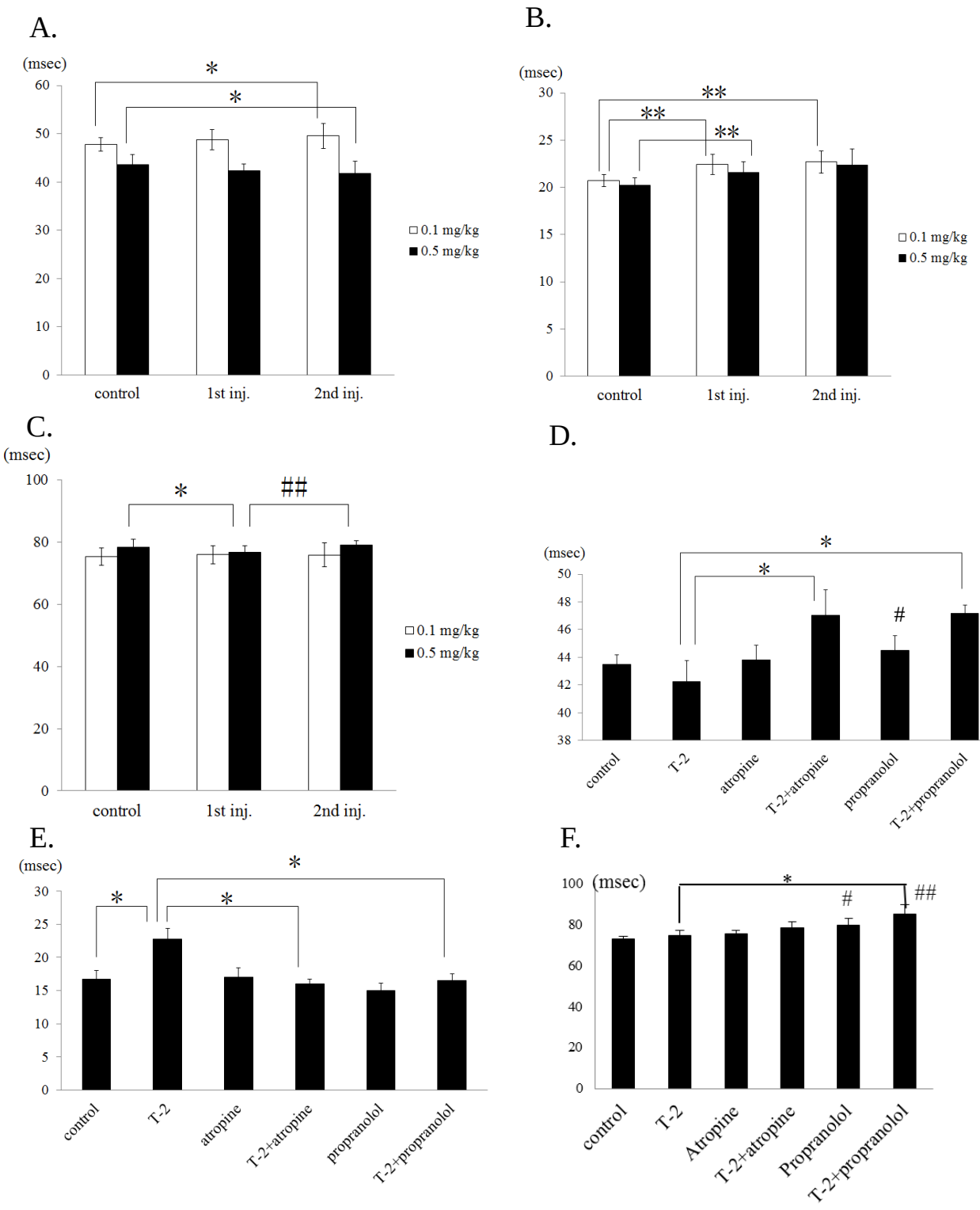

A.
(msec)
60
50
40
30
20
10
0
*
*
control
1st inj.
2nd inj.
0.1 mg/kg
0.5 mg/kg
B.
(msec)
30
25
20
15
10
5
0
**
**
**
control
1st inj.
2nd inj.
0.1 mg/kg
0.5 mg/kg
C.
(msec)
100
80
60
40
20
0
*
##
control
1st inj.
2nd inj.
0.1 mg/kg
0.5 mg/kg
D.
(msec)
50
48
46
44
42
40
38
*
*
#
control
T-2
atropine
T-2+atropine
propranolol
T-2+propranolol
E.
(msec)
30
25
20
15
10
5
0
*
*
*
control
T-2
atropine
T-2+atropine
propranolol
T-2+propranolol
F.
100
(msec)
80
60
40
20
0
*
#
##
control
T-2
Atropine
T-2+atropine
Propranolol
T-2+propranolol

Figure 3.8. Changes in interval components of ECG in response to T-2-injection.
A: PR-interval, B: QRS duration, C: QT-interval, D: PR-interval, E: QRS duration, F: QT-interval. Asterisks in A to C: significant difference (*p<0.05, **p<0.01) from the control before T-2 toxin injection. Number signs in C: significant difference (##p<0.01) between the first and second injection. Asterisks in D to F: significant difference (*p<0.05, **p<0.01) between the "T-2 toxin" and "T-2 toxin+atropine" or "T-2 toxin+propranolol" groups. Number signs in D to F: significant difference (#p<0.05, ##p<0.01) from the control. Data are expressed as means ± SEM.

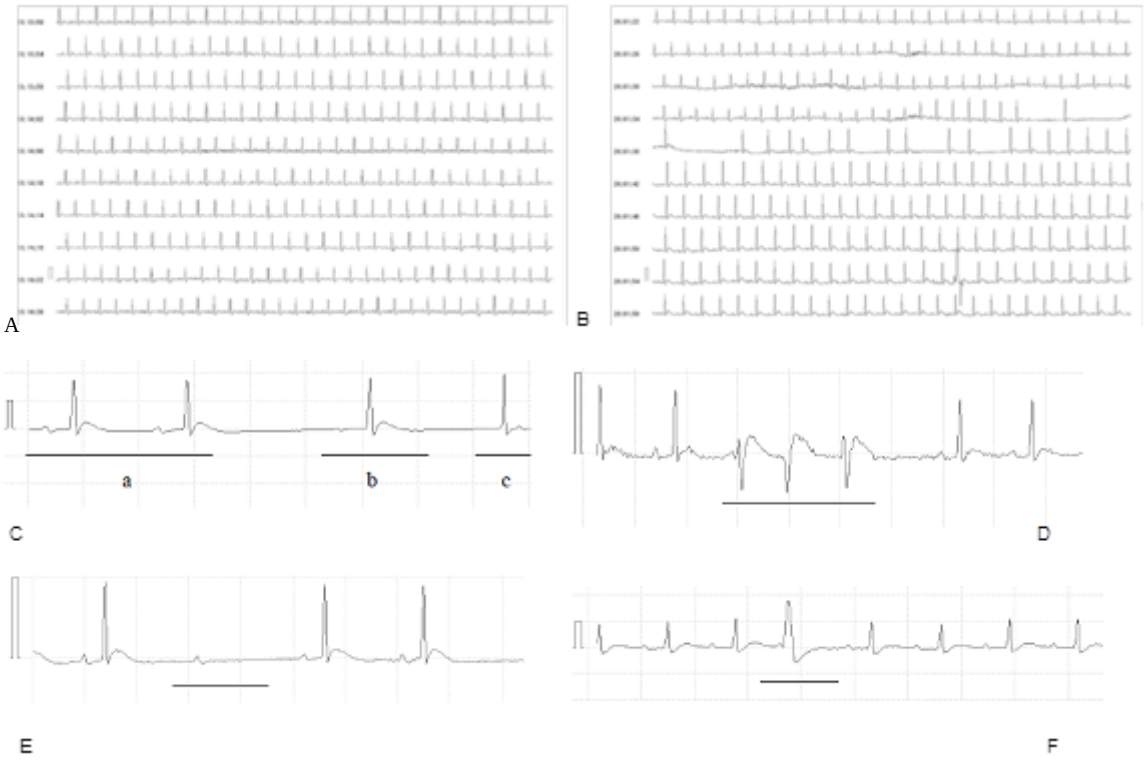
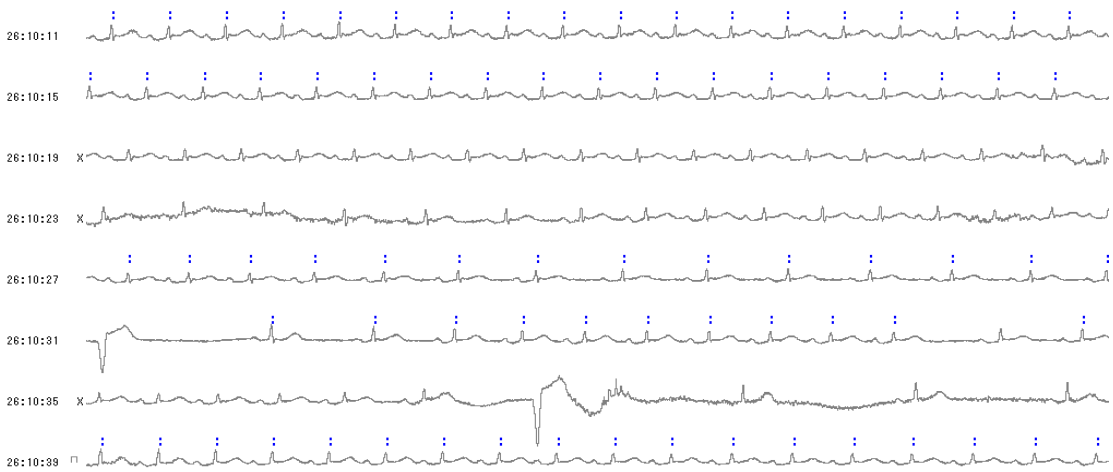


Figure 3.9. Representative records of telemetric electrocardiogram (ECG)
A: Normal ECG pattern before the injection of DON. B-F: Abnormal ECG pattern, including second-degree AV block after the DON-injection (B). C: Normal heart rhythm (a), Atrial bradycardia (b), Supraventricular extrasystole (c) D: Left ventricular extrasystole (short-run type). E: Second-degree AV block (underlined) F: Right ventricular extrasystole (underlined).

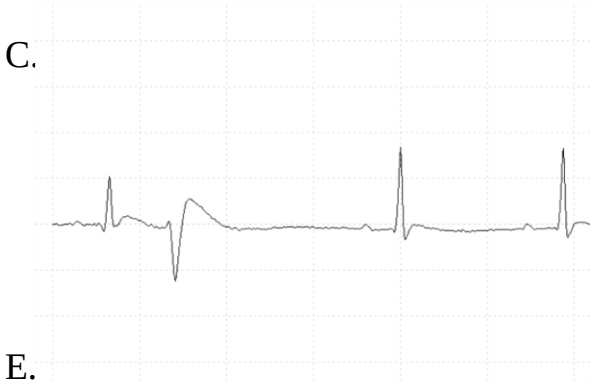
A.


27:00:03
27:00:07
27:00:11
27:00:15
27:00:19
27:00:23
27:00:27

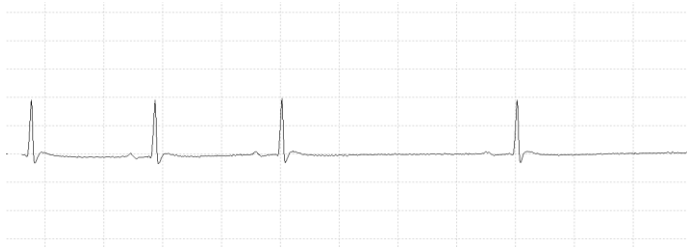
B.


26:10:11
26:10:15
26:10:19
26:10:23
26:10:27
26:10:31
26:10:35
26:10:39

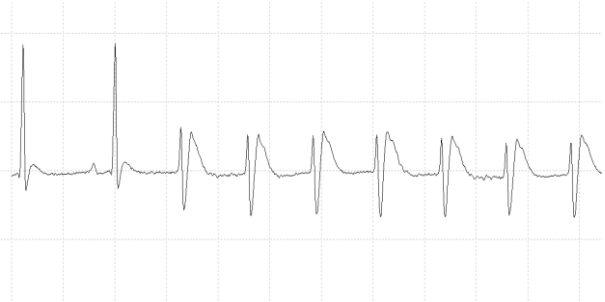
C.



D.



E.



F.

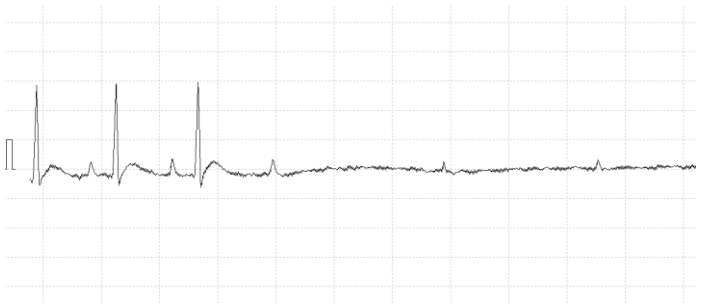


Figure 3.10. Representative records of telemetric electrocardiogram (ECG).
A: Normal ECG pattern before the injection of T-2 toxin, B-F: Abnormal ECG pattern, B: Second-degree AV block and left ventricular extrasystole after the T-2 toxin-injection. C: Left ventricular extrasystole (underlined), D: Normal heart rhythm (a), Atrial bradycardia (b), E: Ventricular tachycardia (underlined), F: Second-degree AV-block (underlined).

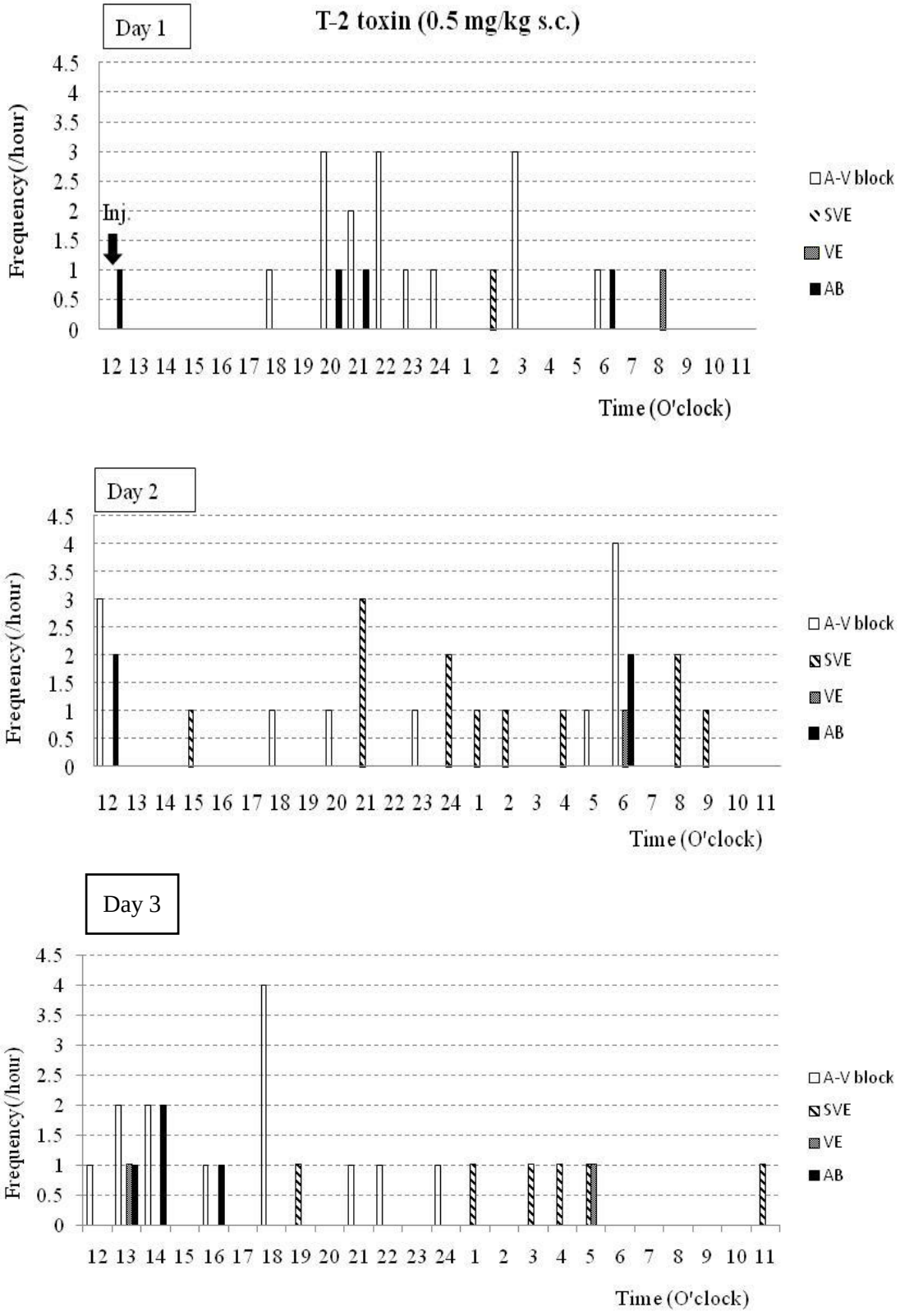

Day 1
T-2 toxin (0.5 mg/kg s.c.)
Frequency(/hour)
Inj.
A-V block
SVE
VE
AB
Time (O'clock)
Day 2
Frequency(/hour)
A-V block
SVE
VE
AB
Time (O'clock)
Day 3
Frequency(/hour)
A-V block
SVE
VE
AB
Time (O'clock)

Figure 3.11. The occurrence of arrhythmia and time-course changes after the administration of T-2 toxin.

Data were obtained from all rats during 3 consecutive days after the 1st administration of T-2 toxin (0.5 mg/kg). AB, sinus bradycardia; A-V block, second-degree atrioventricular block; SVE, supraventricular extrasystole; VE, ventricular extrasystole and ventricular tachycardia.

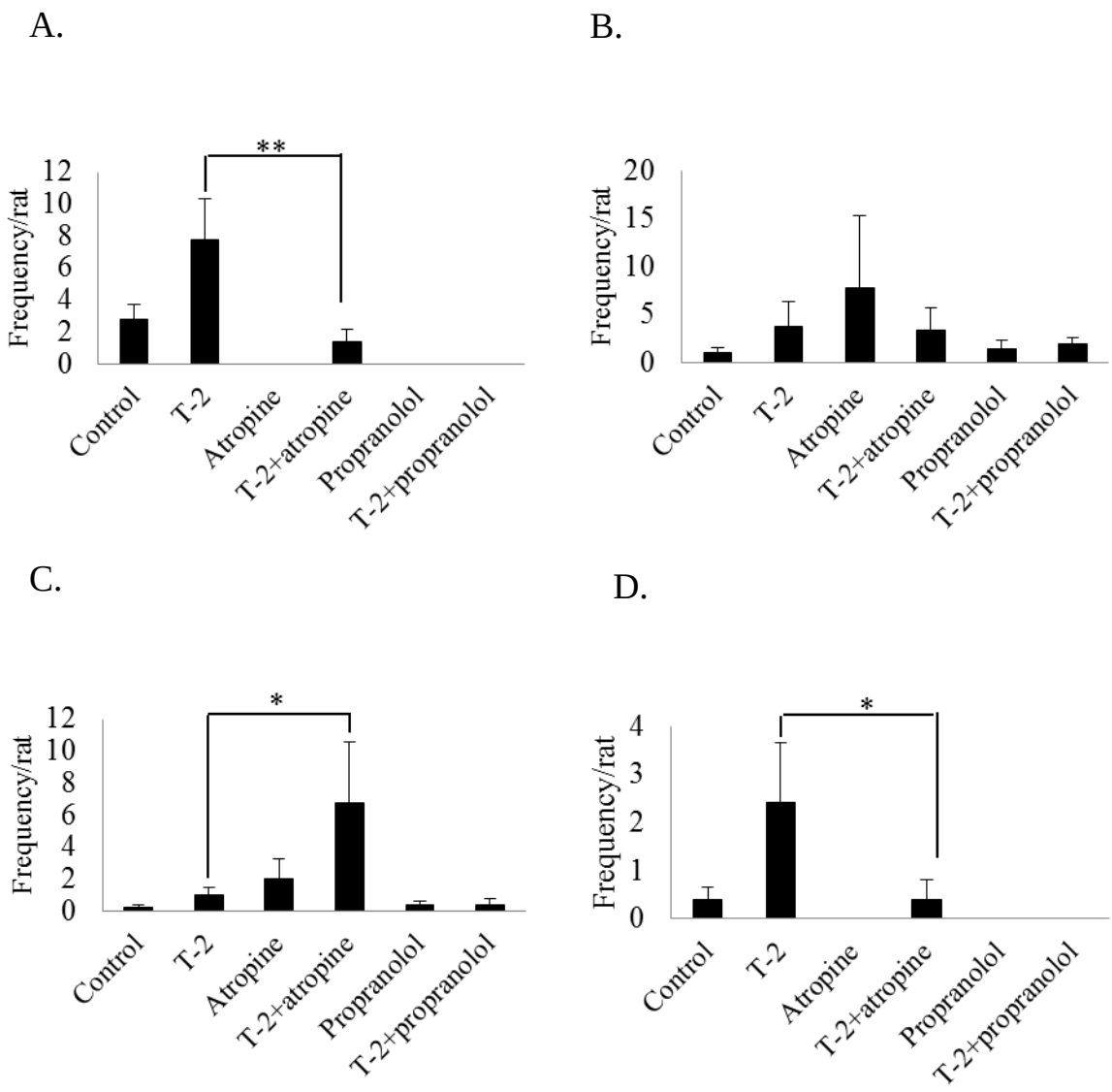


Figure 3.12. Change in occurrence of arrhythmia in response to 0.5 mg/kg-T-2 toxin with or without two blockades (atropine 20 mg/kg/day or propranolol 100 mg/kg/day). A: second-degree atrioventricular block, B: supraventricular extrasystole, C: ventricular extrasystole, D: sinus bradycardia. Asterisks: significant difference (*p<0.05, **p<0.01) between "T-2 toxin" group and "T-2 toxin+atropine" or "T-2 toxin+propranolol" group. Data were based on mean values obtained for each 2 or 3 days and expressed as means ± SEM.

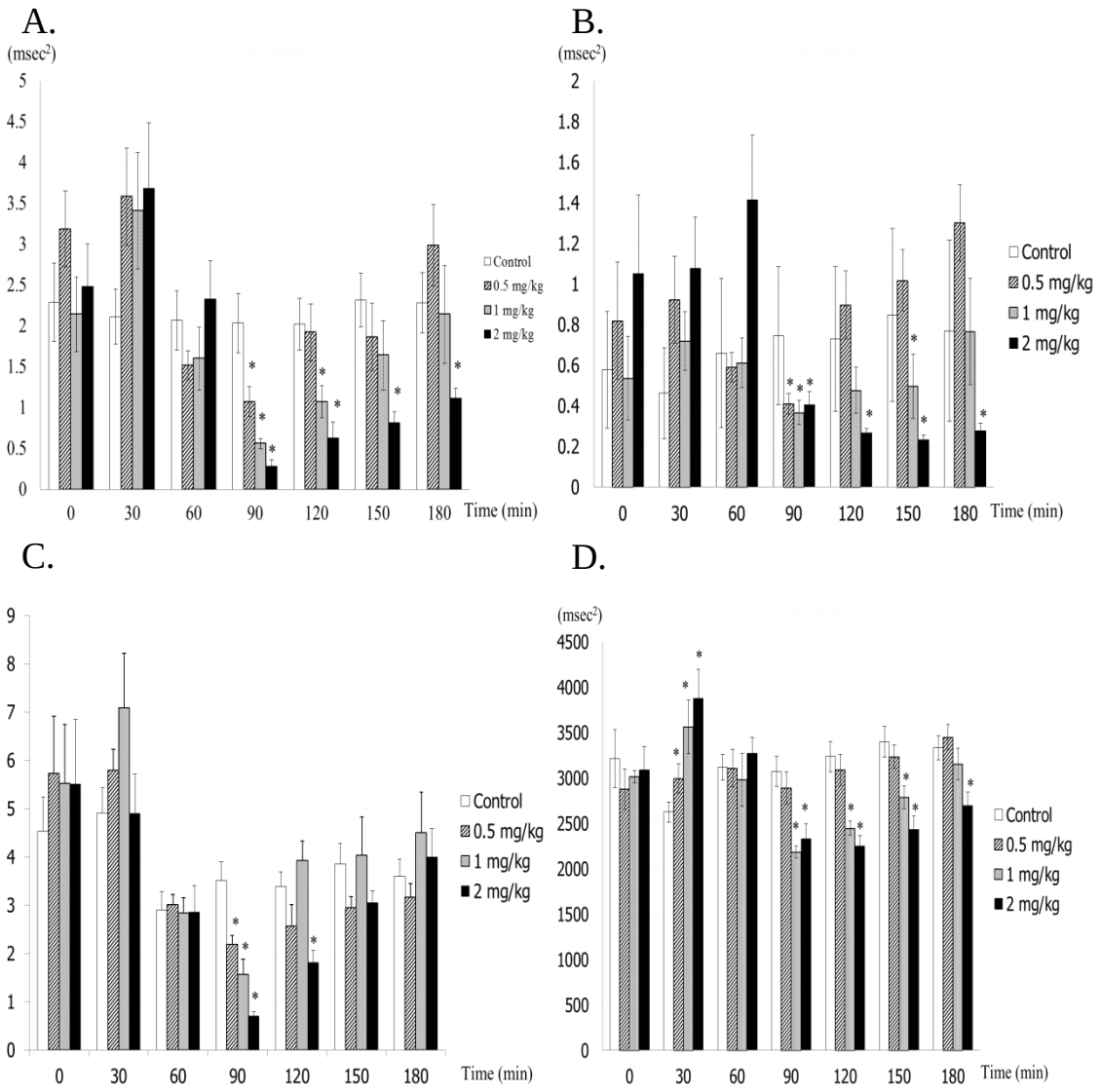


Figure 3.13. Changes in HRV values in response to DON-injection.

A: low-frequency (LF) power, B: high-frequency (HF) power, C: LF/HF ratio, D: total power. Asterisks: significant differences (*p<0.05) from the control. Data are expressed as means ± SEM.

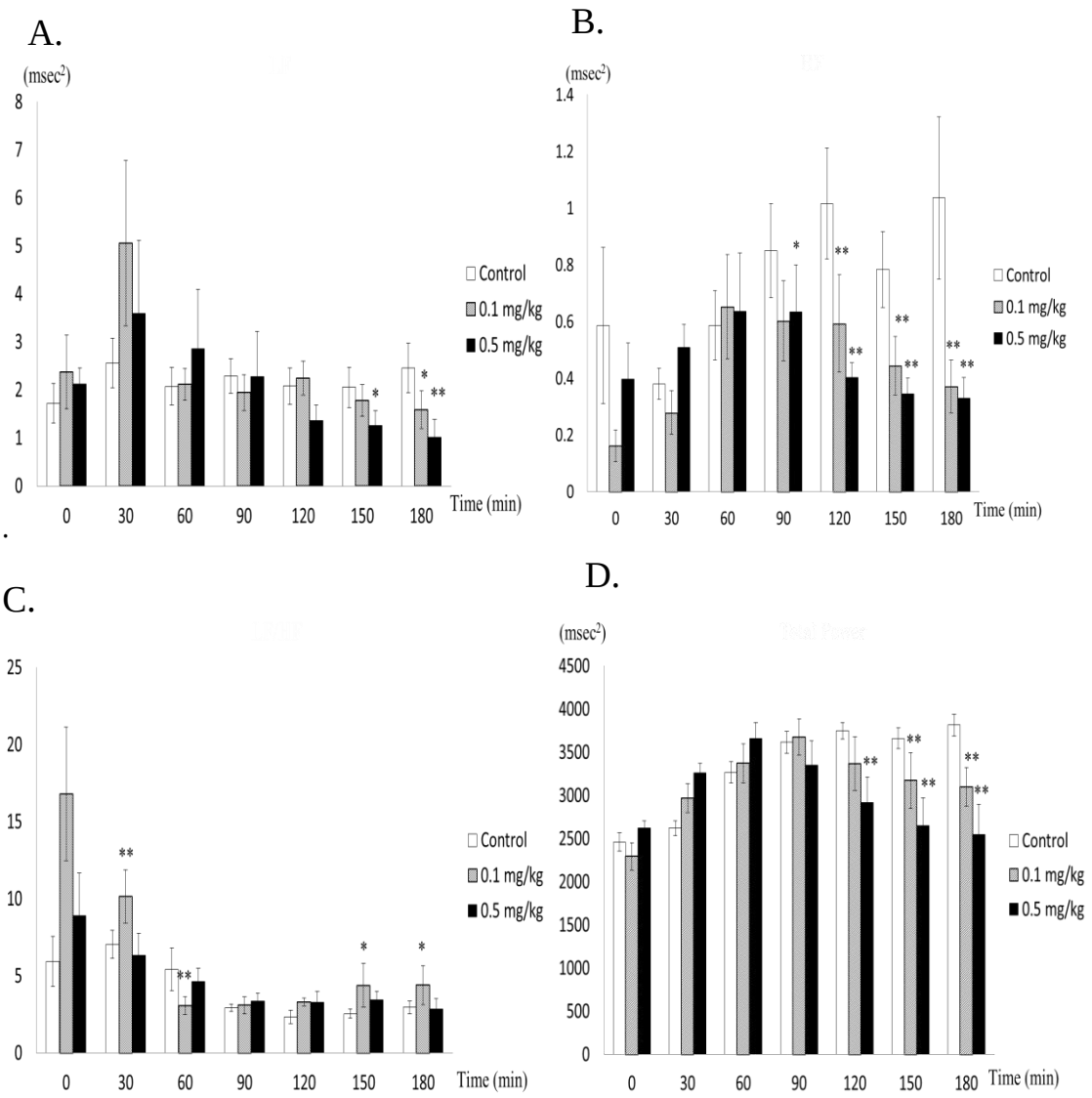


Figure 3.14. Changes in HRV values in response to T-2 toxin-injection.
A: low-frequency (LF) power, B: high-frequency (HF) power, C: LF/HF ratio, D: total power. Asterisks: significant differences (*p<0.05, **p<0.01) from the control. Data are expressed as means ± SEM.

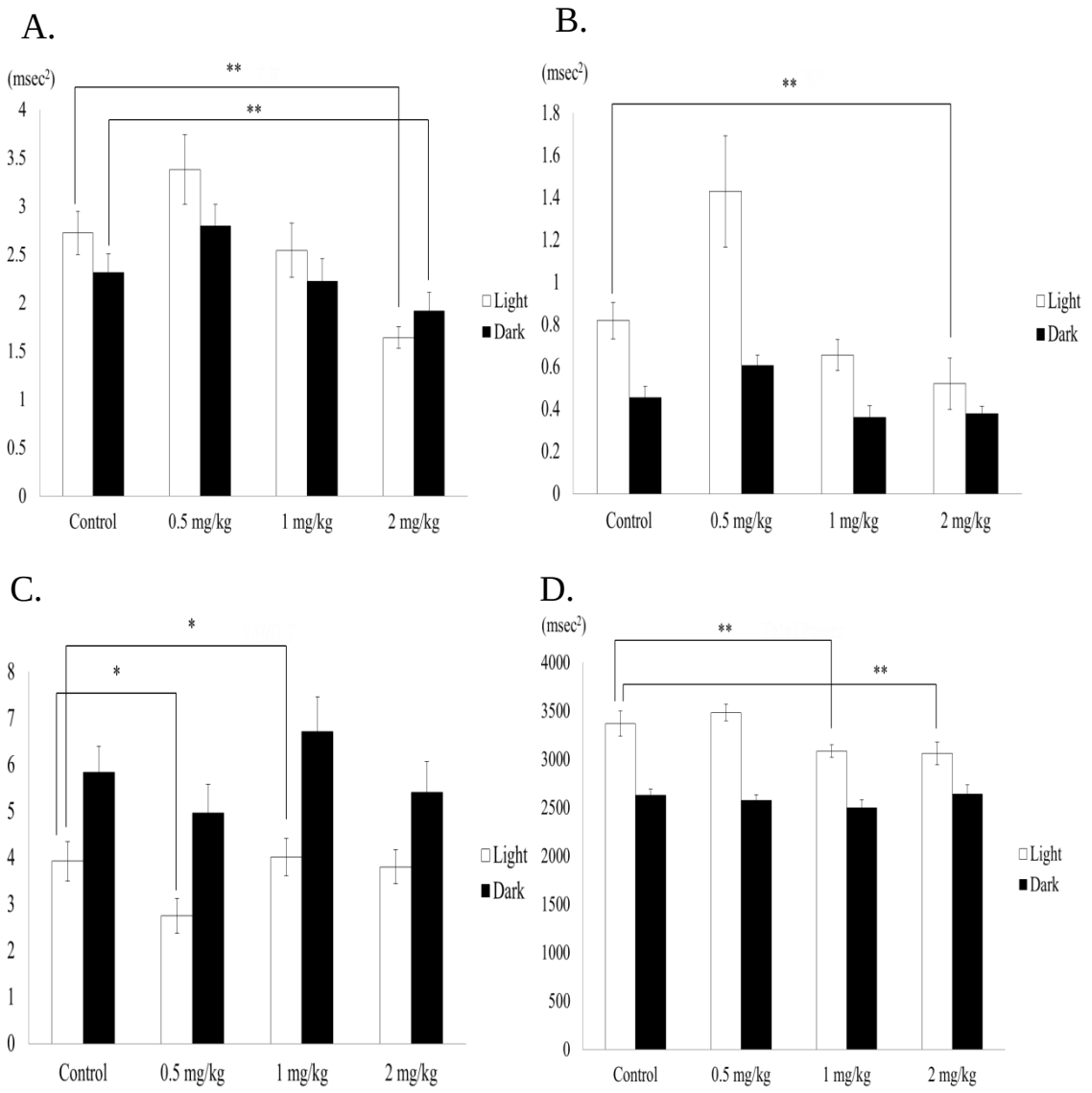


Figure 3.15. Changes in HRV values in response to DON-injection.

A: low-frequency (LF) power, B: high-frequency (HF) power, C: LF/HF ratio, D: total power. *, **: significant differences (*p<0.05, ** p<0.01) from the control. Data were based on mean values obtained for each one day and expressed as means ± SEM.

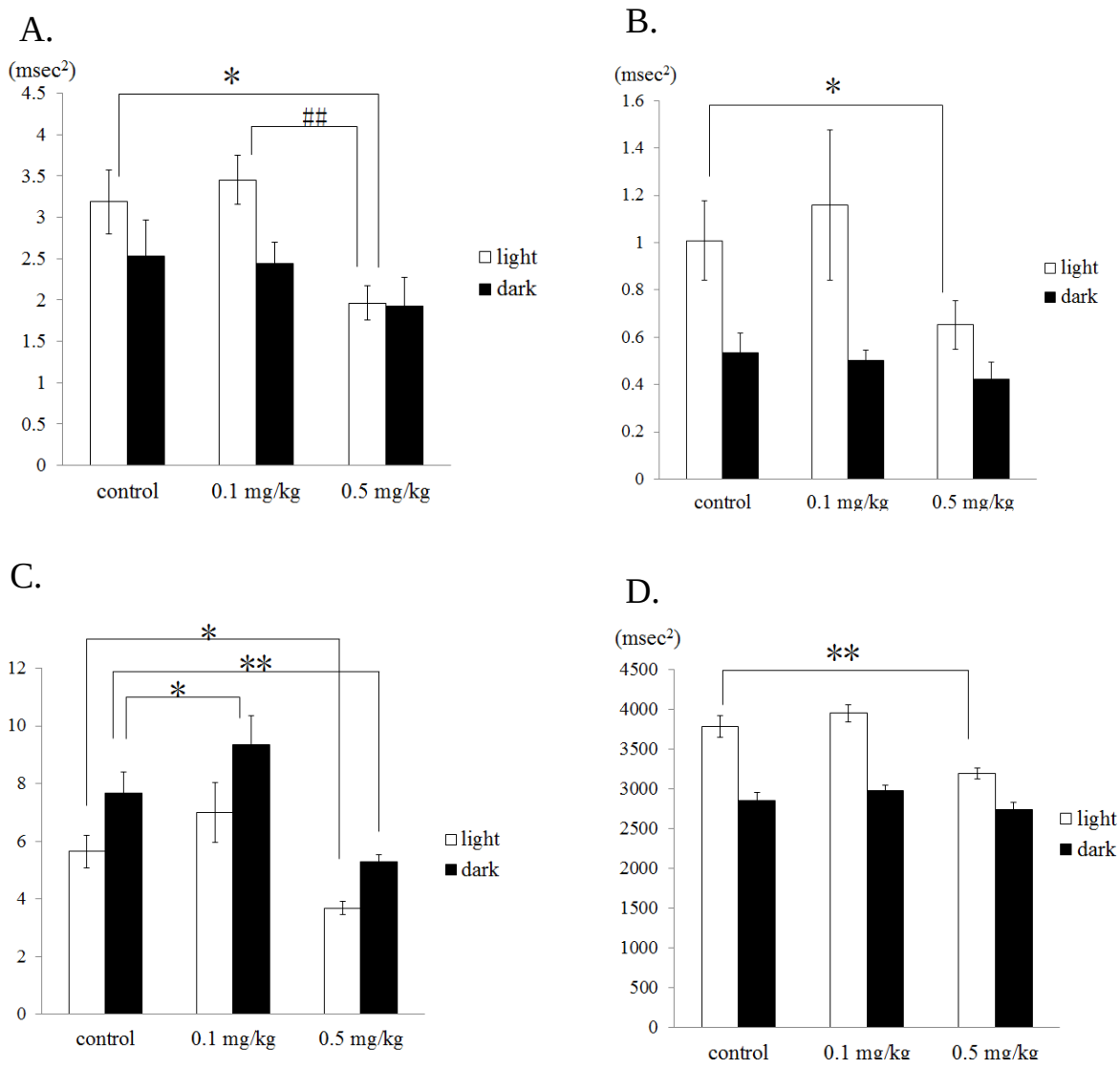


Figure 3.16. Changes in the value of power spectrum of heart rate variability in response to T-2-injection.

A: low-frequency (LF) power, B: high-frequency (HF) power, C: LF/HF ratio, D: total power. Asterisks: significant differences (*p<0.05, ** p<0.01) from the control. Number signs: significant difference (##p<0.01) from the 0.1 mg/kg-T-2 group. Data were based on mean values obtained for each 3 days and expressed as means ± SEM.

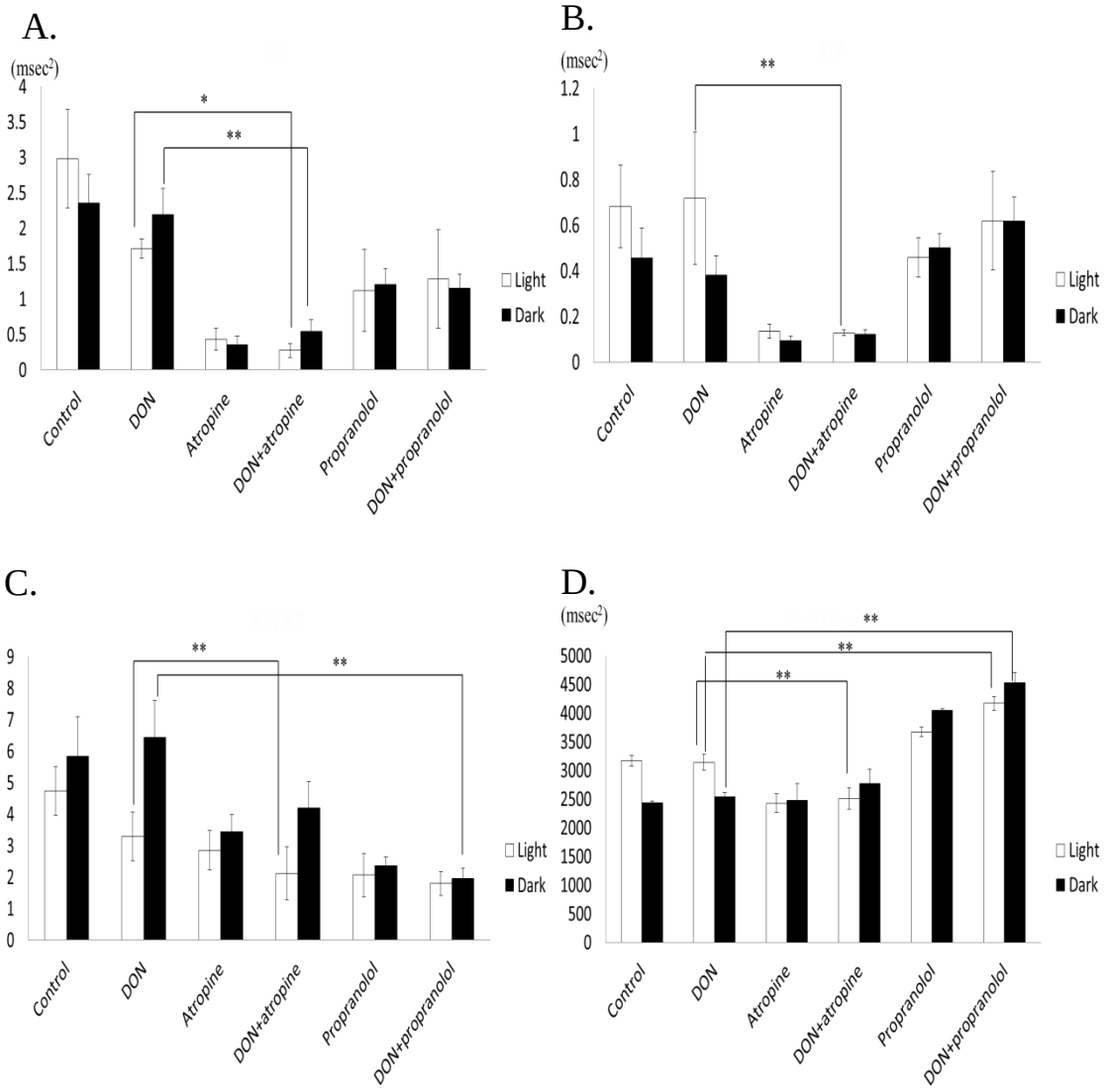


Figure 3.17. Changes in HRV values response to 2 mg/kg-DON with blockades (atropine 20 mg/kg/day or propranolol 100 mg/kg/day).

A: low-frequency (LF) power, B: high-frequency (HF) power, C: LF/HF ratio, D: total power. Asterisks: significant difference (*p<0.05, **p<0.01) from the 2 mg/kg-DON administration group. Data were based on mean values obtained for each one day and expressed as means ± SEM.

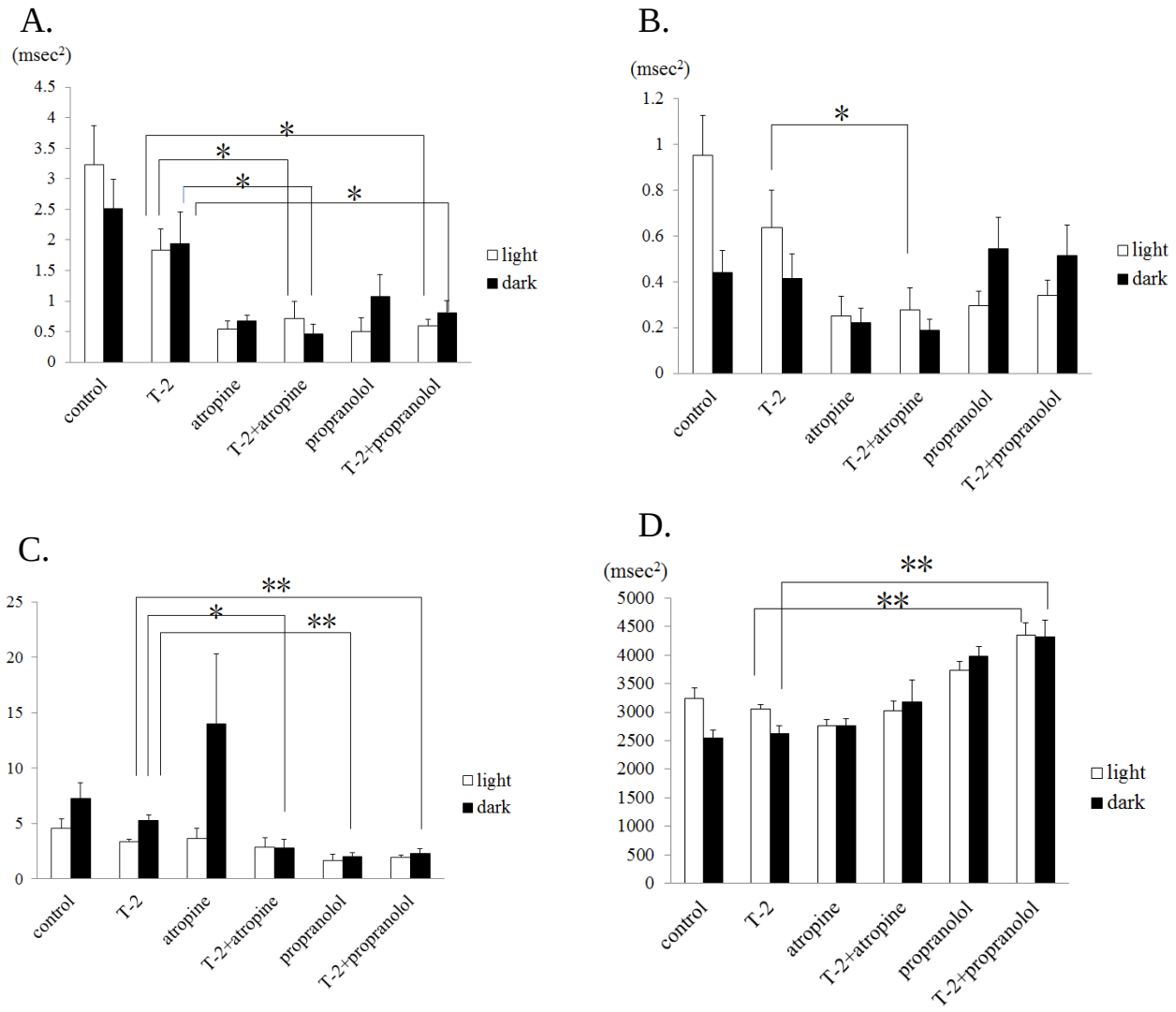


Figure 3.18. Changes in the value of power spectrum of heart rate variability in response to 0.5 mg/kg-T-2 toxin with blockades

A: low-frequency (LF) power, B: high-frequency (HF) power, C: LF/HF ratio, D: total power. Asterisks: significant difference (*p<0.05, **p<0.01) from the 0.5 mg/kg-T-2 toxin administration group. Data were based on mean values obtained for each 3 days and expressed as means ± SEM.

CHAPTER 4

DYNAMIC CARDIOVASCULAR CHANGES AND BLOOD OXIDATIVE STRESS INDUCED BY T-2 TOXIN

4.1 Introduction

In the previous chapter, cardiac functions were measured via telemetry method which served a good source for studies on HR change, arrhythmia occurrences and more commonly ANS activity. However, this method cannot provide a measurement of the dynamic circulatory function like stroke volume (SV), cardiac output (CO), ejection fraction (EF), etc. In this chapter, the echocardiographic study was performed to obtain changes in these parameters in rats with T-2 toxin. Furthermore, oxidative stress and anti-oxidant potential in serum was measured to investigate a relationship of vascular changes that might receive an influence from reactive oxygen species (ROS) induced by T-2 toxin administration.

According to chapter 3, T-2 toxin's toxicity persisted as long as 72 h while DON toxicity lasted utmost 6 h after subcutaneous administration was reported. In the previous chapter, DON was not used in this chapter because of its rapid decay, within a day, in the toxicity. From the previous chapter, the occurrence of arrhythmia was identified mostly at day 2 following the injection and therefore 48 h was chosen in order to investigate the circulatory changes following T-2 toxin administration. There has been many reports about acute toxicity of T-2 toxin on vascular effects following T-2 toxin administration by direct measurement with probes that were implanted in the vessels in various species like cats, swine, guinea pigs and rats, etc. (Sato et al., 1975, Weaver et al., 1978, Feuerstein et al., 1985, Anna-Leena et al., 1986) . In the present study, the echocardiogram was used for assessing the cardiovascular functional changes because of its recent advancements in the ultrasound technique that allows thorough echocardiographic examination even in small laboratory mammals (Watson et al., 2004).

Furthermore, this method has been established as a safe, reproducible, and accurate assessment of cardiac anatomy, hemodynamics, and cardiac function.

Chaudhari et al., (2009) reported that T-2 toxin induced oxidative stress, DNA damage and triggered apoptosis pathway. The oxidative stress is a result from ROS production which is the metabolites of molecular oxygen (O_2) that have higher reactivity than O_2. The ROS can include unstable oxygen radicals such superoxide radical ($O_2^{\cdot -}$) and hydroxyl radical ($HO^{\cdot}$), and nonradical molecules like hydrogen peroxide (H_2O_2). In this study, d-ROMs test which is served as a method to detect ROS was performed by the use of Fenton's reaction principle, that detects hydroperoxide (ROOH) level which is considered as an indicator of oxidative attack of ROS. In the same time, a protective response in the body against an excessive oxidative stress can be evaluated by BAP test which is the technique that measures the ferric reducing ability.

The aim of this study is to investigate circulatory impairments at heart and vascular levels induced by T-2 toxin. Furthermore, an attempt was conducted to find a relationship of these impairments with the oxidative stress induced by T-2 toxin which may account for the toxic mode of T-2 toxin in the cardiovascular system.

4.2 Materials and Methods

Animal and housing

The experiment was performed using 15 male Wistar rats purchased from Japan SLC, Inc. (Shizuoka, Japan) at 8 weeks of age and having the body weights of 230-250 g at 10 weeks of age. Each rat was maintained with *ad libitum* access to food and water in an individual cage controlled lighting (light-dark cycle, light=12:00-24:00,

dark=24:00-12:00) and temperature conditions (24^{o}C). All rats were fully adapted to these breeding environments during experiments. All experiments were conducted in accordance with the Animal Experimentation Guidelines of the University of Tokyo and approved by the institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences at the University of Tokyo.

Toxin preparation

The preparation of T-2 toxin was done in accordance to the method described in Chapter 2.

Injection protocol

All rats were randomly divided into three groups to achieve 5 animals per group. A single injection of olive oil which was served as vehicle control and T-2 toxin at doses of 0.1 and 0.5 mg/kg was administered subcutaneously 48 h prior echocardiogram measurement in a different group of treatment. All the injections were performed at 12:00.

Echocardiographic studies and data analysis

At 48 h after the administration of vehicle or T-2 toxin, each rat was assigned for the echocardiographic measurement under the anesthetic condition with urethane (1-2 g/kg, i.p.) and in a supine position. The current ultrasound technology (VisualSonics, Vevo2100) having a high level of resolution (12-MHz probe) was used to measure echocardiographic parameters based on the American Society for Echocardiography Guidelines (Schiller et al., 1989). M-mode and 2-dimensional

(B-mode) echocardiography images were obtained in the parasternal long- and short-axis views as shown in Figure 4.1. The thickness of the intraventricular septum and posterior wall, and left ventricular (LV) dimensions were determined at the tips of the papillary muscle. The SV, CO, EF and fractional shortening (FS) were obtained at area of the heart. Color Doppler mode and pulse wave Doppler mode were performed at the level of the common carotid artery and the femoral artery as shown in Figure 4.2. Peak systolic velocity (PSV), end diastolic velocity (EDV) and mean velocity (MV) were measured from the common carotid artery and the femoral artery and assigned for calculation of the pulsatility index (PI) and resistive index (RI) as shown in the equations as follows; PI = (PSV-EDV)/MV and RI = (PSV-EDV)/PSV.

Blood collection

The rats that received echocardiographic measurement were euthanized by cutting the common carotid artery to collect blood sample without anticoagulant substances. The blood sample was placed in an ice box for at least 30 min to obtain the serum. This serum is used for measurements of oxidative stress (d-ROMs) and anti-oxidant potential (BAP) (Cesarone *et al.*, 1999, Cornelli *et al.*, 1999, Alberti *et al.*, 2000, Trotti *et al.*, 2001, Gerardi *et al.*, 2002).

d-ROMs and BAP test

These tests were performed by using Free Radical Elective Evaluator (Wismerll Co. Ltd. Bunkyo-ku, Tokyo). As mentioned above, d-ROMs (Diacron-Reactive Oxygen Metabolites) test was used for the detection of ROS while BAP (Biological Antioxidant Potential) test was for the measurement of anti-oxidant

potential. A change of color after mixing serum with the reagents was measured as optical density (OD).

Statistical Analysis

All the results in the present studies were statistically evaluated by one-way non-repeated-measures analysis of variance (one-way non-repeated-measures ANOVA). In addition, Dunnett's test was used to test significant differences between control and treatment groups for all parameters. Fisher's test was assigned to test significant differences between treatment groups for all parameters. Differences with p values less than 0.05 were regarded as significant.

4.3 Results

Heart rate (HR) changes

Changes in the HR at 48 h after the administration at 0.1 mg/kg and 0.5 mg/kg of T-2 toxin were shown in Figure 4.3A. In the 0.5 mg/kg-T-2 toxin showed a trend of increase in HR (P=0.13) when compared with the control and the 0.1 mg/kg-T-2 toxin group, while no significant differences were identified.

Stroke volume (SV)

Changes in the SV observed at 48 h after the administration with vehicle or T-2 toxin (0.1 and 0.5 mg/kg) are represented in Figure 4.3B. Although the SV was slightly decreased in the 0.1 mg/kg-T-2 toxin group, no significant differences were observed either the 0.1 or 0.5 mg/kg-T-2 toxin group compared with the control.

Cardiac output (CO)

Changes in the CO were represented in Figure 4.3C. The CO tended to increase in the 0.5 mg/kg-T-2 toxin group when compared with the control group without any significant differences. However, the significant change was identified in a comparison between low dose and high dose injection, i.e., the CO in the 0.5 mg/kg-T-2 toxin group significantly increased when compared with the 0.1 mg/kg-T-2 toxin-group ($p<0.05$).

Ejection fraction (EF)

The T-2 toxin administration did not induce any significant changes in EF (Figure 4.3D), although the EF in the 0.1 mg/kg-T-2 toxin group tended to be slightly increased from the control group.

Fractional shortening (FS)

There was no significant change in FS as shown in Figure 4.3E, while a tendency for dose-dependent increase could be observed.

Blood flow and vascular resistance in the femoral artery

Hemodynamic parameters that were observed in the peripheral vascular level are EDV, PSV, MV, PI and RI. At each dose of injection, no significant difference was observed in all parameters (Figure 4.4A-E), although in the 0.5 mg/kg-T-2 toxin group, the EDV, PSV and MV were tended to be higher than those in the control group (Figure 4.4A-C), and the PI and RI were shown to be lower at the same dose (Figure 4.4D, E).

Blood flow and vascular resistance in the common carotid artery

Changes in the EDV, PSV, MV, PI, and RI were also measured from the common carotid artery (Figure 4.5A-E). The MV in the 0.5 mg/kg-T-2 toxin was significantly increased from the control ($p<0.05$) and also from the 0.1 mg/kg-T-2 toxin group ($p<0.05$) (Figure 4.5C). In addition, the PI in the 0.5 mg/kg-T-2 toxin was significantly lower than that in the 0.1 mg/kg-T-2 toxin -group ($p<0.05$), although there was no significance when compared with the control group (Figure 4.5D). In the EDV, PSV and RI, no significant differences were identified in any group of injection (Figure 4.5A, B, and E).

Oxidative stress level (d-ROMs Test)

Changes in the oxidative stress level in the serum were shown in Figure 4.6A. The administration of 0.1 and 0.5 mg/kg-T-2 toxin both induced a significant increase of d-ROMs values in a dose-dependent manner (one-way non-repeated-measures ANOVA) when compared with the vehicle injection ($p<0.05$ and $p<0.01$, respectively).

Anti-oxidant potential (BAP test)

There was no significant difference of anti-oxidant potential change in any dose of T-2 toxin administration when compared with the control as shown in Figure 4.7B.

d-ROMs/BAP ratio

Changes in d-ROMs/BAP ratio that indicates a balance between ROS production and anti-oxidant ability are shown in Figure 4.7C. The d-ROMs/BAP ratio

was increased in a dose-dependent manner (one-way non-repeated-measures ANOVA) was also identified. In the 0.5 mg/kg-T-2 toxin group, this ratio was significantly higher than that in the control group as shown in Figure 4.7C ($p<0.05$).

4.4 Discussion

The previous reviews described that the acute toxicity of T-2 toxin was revealed as an increase in total peripheral resistances following with a decrease in CO in the swine, guinea pig and rat (Feuerstein et al., 1985, Lorenzana et al., 1985, Siren and Feuerstein, 1986, Lundeen et al., 1986). In the present study, the effect of T-2 toxin at 48 h after the administration was evaluated as the increase of HR, SV and CO in the higher dose of T-2 toxin. Especially, the CO in the higher dose (0.5 mg/kg) of T-2 toxin showed the significant difference compared with the lower dose of T-2 toxin (0.1 mg/kg). The increase in CO at 0.5 mg/kg in the present study might be affected by an increase in HR, because the CO is expressed by the following formula; CO = SV x HR and no substantial change in SV. The results in chapter 2 and 3, in which the increase in the HR was shown from 90min-72h after the administration of T-2 toxin, may be associated with the increase in the CO. The increase in CO might be considered as an important physiological function to protect the body from severe hypotensive condition since the systemic blood pressure is expressed by product of CO and peripheral resistance. In fact, in this study the tendency for decrease in PI and RI were identified in the femoral artery in rats with the higher dose of T-2 toxin.

Both the carotid and femoral vessels exhibited increased responses in EDV, PSV, and MV at the high dose (0.5 mg/kg) of T-2 toxin injection. In general, the increase in EDV indicates the left ventricular dysfunction (Saraiva et al., 2007), and the

high PSV value suggests the stenosis in the vessel in which usually used as diagnosis for stenosis in the carotid artery in humans (Gaitini and Soudack, 2005). All hemodynamic parameters in the femoral artery which is represented as hindquarter vessel did not significantly change from the control group. The MV in the common carotid artery was significantly increased following to high dose of T-2 toxin administration in comparison with the control and the low dose of T-2 toxin group. The tendency for increase of PSV and MV in the common carotid artery might reflect the increase in CO in rats with high dose of T-2 toxin. Yarom et al. (1987) reported that T-2 toxin caused endothelial cell damage, accumulation of basement membrane-like material in the intima, and activation with proliferation of smooth muscle cells in the rat aorta at day 2 following the administration of single dose 2 mg of T-2 toxin. A similar vascular change may be induced in the common carotid artery.

In the present study, it was demonstrated that T-2 toxin stimulates ROS production (Figure 4.7A) which may later accelerate the apoptosis pathway (Bouaziz et al., 2009, Wu et al., 2011, Fang et al., 2012). Recent studies on T-2 toxin that induces ROS mostly focus on the effects on a particular organ, cell and molecular level. However, a main objective in the present study was to identify effects of T-2 toxin at a whole body level and the oxidative stress level in serum was significantly higher in the T-2 toxin group than the control group. This indicates that the ROS production systemically occurred in rat that received T-2 toxin. In addition, anti-oxidant potential was also measured to compare a balance between ROS production and anti-oxidant effect inside the body. There was no significant change in anti-oxidant level between vehicle and T-2 toxin treated groups. Therefore, the balance (d-ROMs/BAP ratio) between oxidative stress and anti-oxidative stress exhibited the marked increase in the

oxidative stress. It is feasible that ROS induced by T-2 toxin caused cardiac damages and vascular alteration in which later leaded to cardiovascular dysfunction.

4.5 Summary

The dynamic changes in the cardiovascular system were investigated by the aid of echocardiograph and the blood oxidative stress was measured in rats with administration of T-2 toxin (0.1 and 0.5 mg/kg, s.c.). The echocardiographic observation explored the alterations of cardiovascular functions showing the tendency for the increase in the CO, EDV and PSV and significant increase of MV in the observation at 48 h after the toxin injection. Furthermore, the oxidative stress, d-ROMs values, in serum was significantly increased in a dose-dependent manner at 48 h after the toxin injection. Results of this study show that oxidative stress might be a part of the underlying mechanism by which T-2 toxin causes the cardiovascular dysfunction.

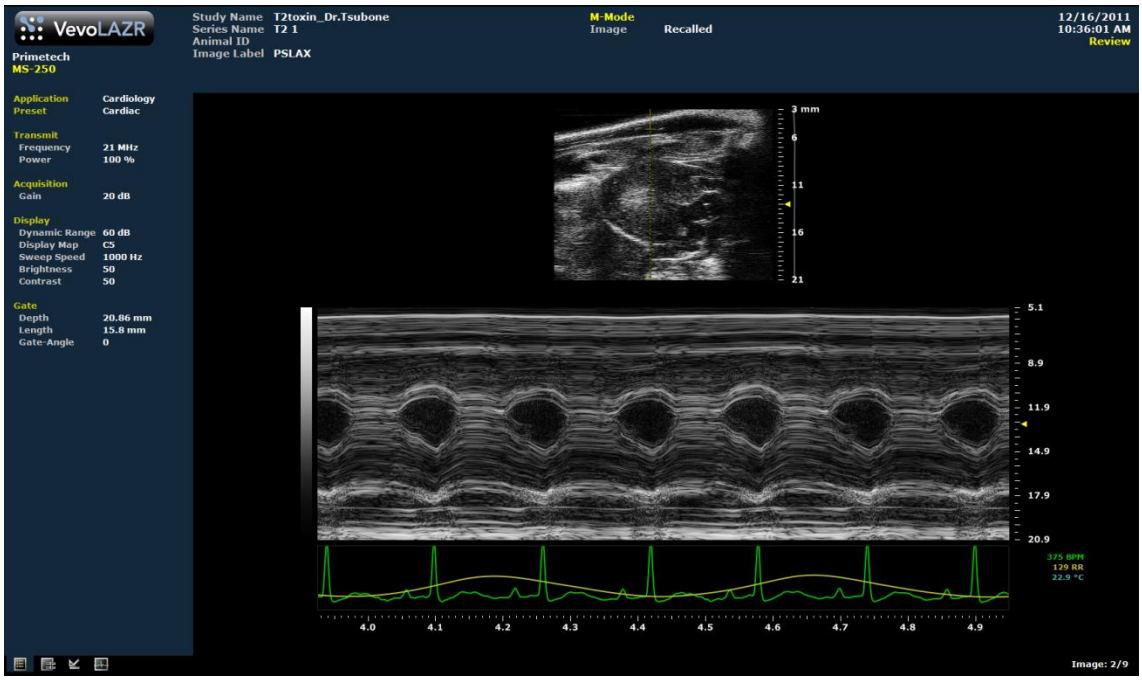


Figure 4.1 A representative record of echocardiogram in a rat

The upper image: B-mode measurement. The middle second: M-mode measurement in parasternal short-axis at the midventricular level. The lowest records: electrocardiogram and respiratory cycle.

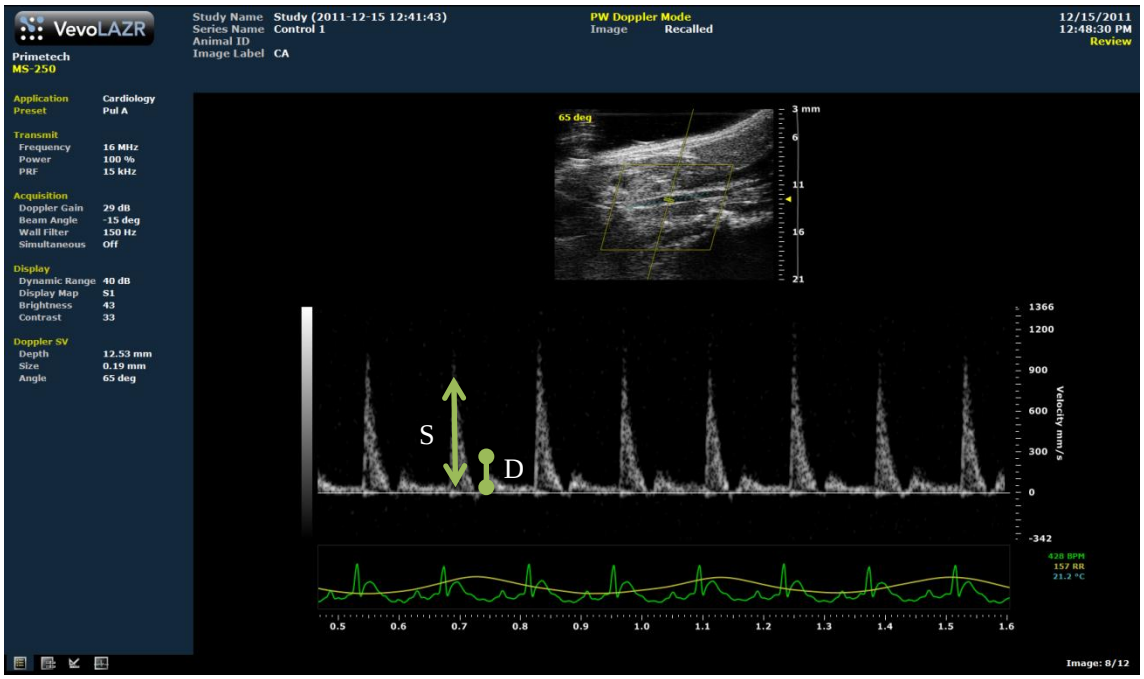
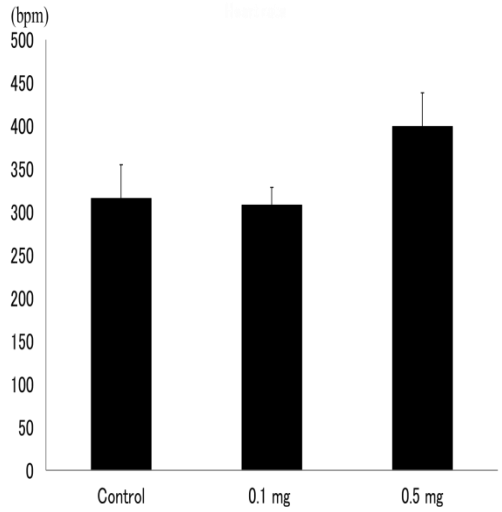
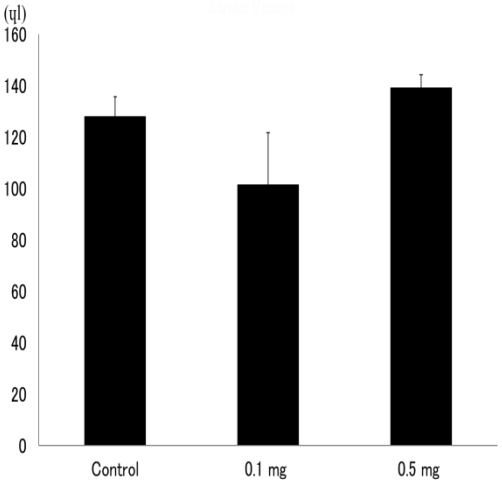


Figure 4.2 A representative record of blood flow in the common carotid artery
The upper image: B-mode measurement of the common carotid artery. The middle second: Pulsed wave Doppler display of the common carotid artery in a rat with administration of T-2 toxin. The systolic and end-diastolic velocities were shown as the alphabet “S” and “D”, respectively. The lowest records: electrocardiogram and respiratory cycle.

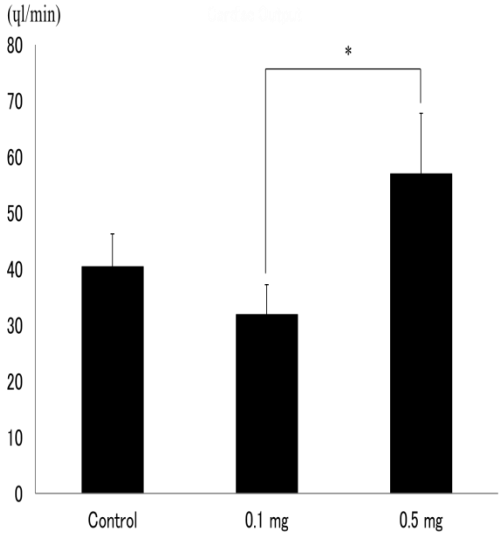
A.


(bpm)
500
450
400
350
300
250
200
150
100
50
0
Control
0.1 mg
0.5 mg

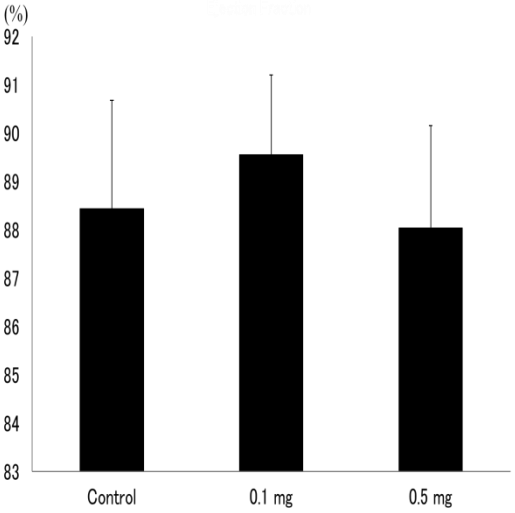
B.


(ql)
160
140
120
100
80
60
40
20
0
Control
0.1 mg
0.5 mg

C.


(ql/min)
80
70
60
50
40
30
20
10
0
*
Control
0.1 mg
0.5 mg

D.


(%)
92
91
90
89
88
87
86
85
84
83
Control
0.1 mg
0.5 mg

E.

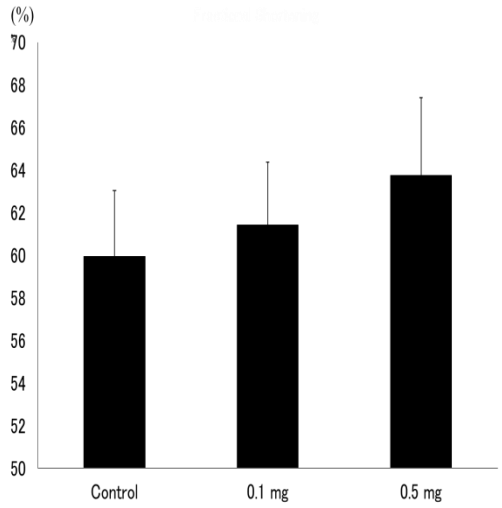
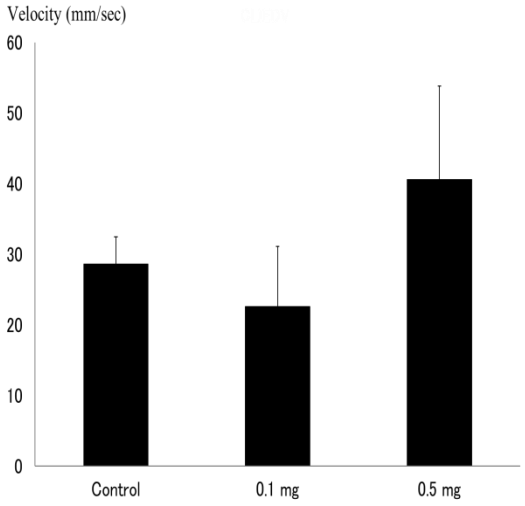

(%)
70
68
66
64
62
60
58
56
54
52
50
Control
0.1 mg
0.5 mg

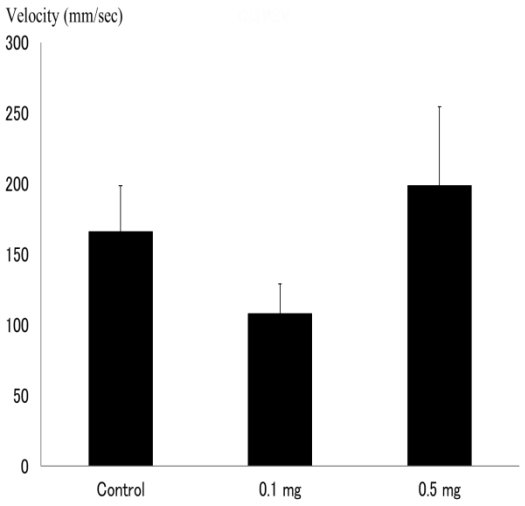
Figure 4.3 Effects of T-2 toxin on hemodynamic parameter in rats at 48 h following subcutaneous administration of the toxin.

A: Heart rate (HR), B: Stroke volume (SV), C: Cardiac output (CO), D: Ejection fraction (EF), E: Fractional shortening (FS). The asterisk shows a significant difference at $p<0.05$ (*) from the 0.1 mg/kg-T-2 toxin group. Data are expressed as means $\pm$ SEM.

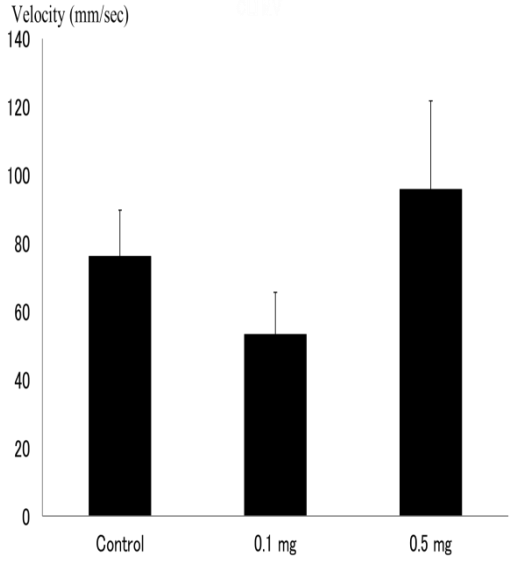
A.


Velocity (mm/sec)
60
50
40
30
20
10
0
Control
0.1 mg
0.5 mg

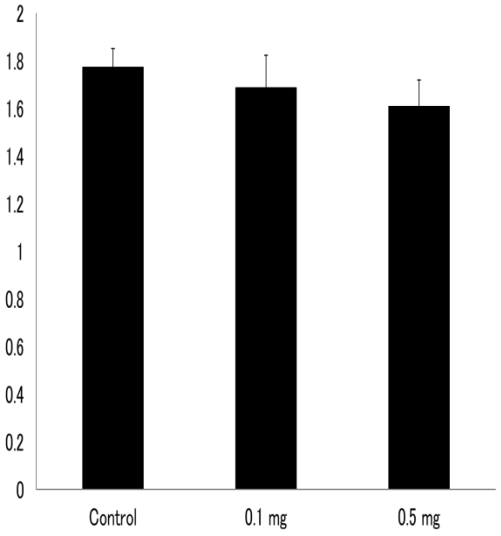
B.


Velocity (mm/sec)
300
250
200
150
100
50
0
Control
0.1 mg
0.5 mg

C.


Velocity (mm/sec)
140
120
100
80
60
40
20
0
Control
0.1 mg
0.5 mg

D.


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
0.1 mg
0.5 mg

E.

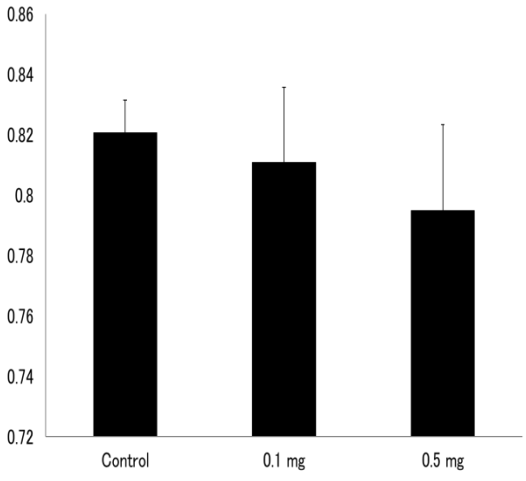
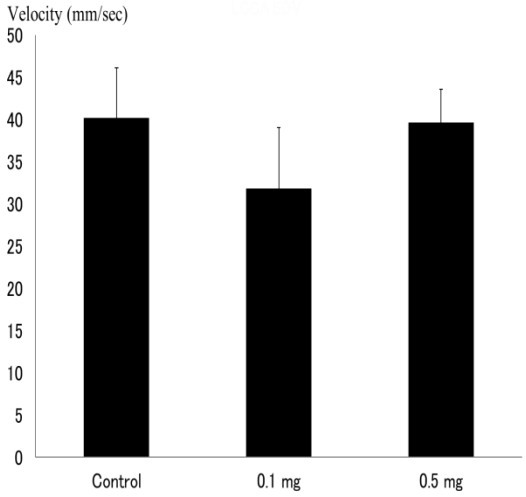
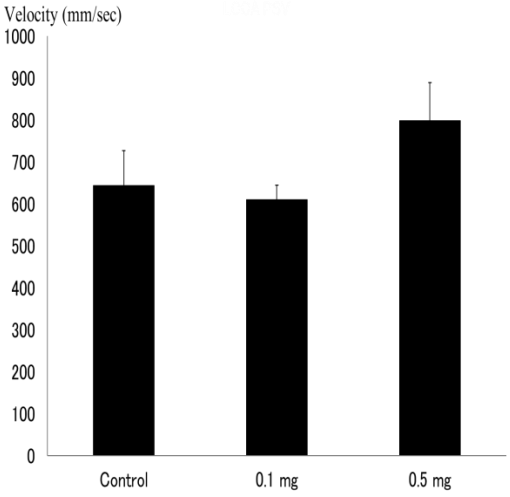

0.86
0.84
0.82
0.8
0.78
0.76
0.74
0.72
Control
0.1 mg
0.5 mg

Figure 4.4 Effects of T-2 toxin on hemodynamic parameters recorded from the femoral artery in rats at 48 h following subcutaneous administration of the toxin.
A: End-Diastolic Velocity (EDV), B: Peak Systolic Velocity (PSV), C: Mean Velocity (MV), D: Pulsatility Index (PI), E: Resistance Index (RI). Data are expressed as means ± SEM.

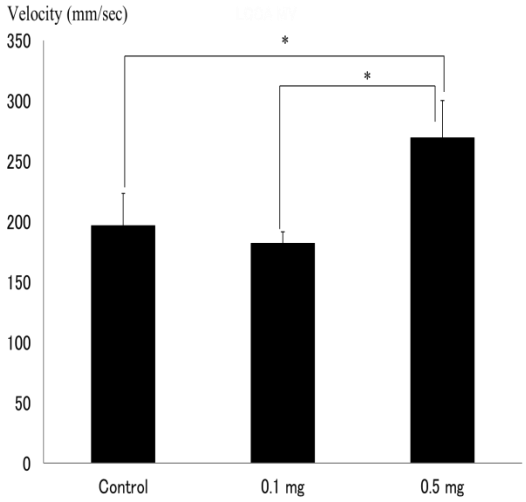
A.


Velocity (mm/sec)
50
45
40
35
30
25
20
15
10
5
0
Control
0.1 mg
0.5 mg

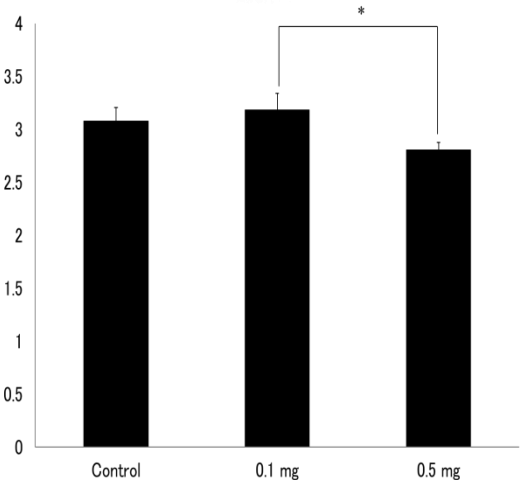
B.


Velocity (mm/sec)
1000
900
800
700
600
500
400
300
200
100
0
Control
0.1 mg
0.5 mg

C.


Velocity (mm/sec)
350
300
250
200
150
100
50
0
*
*
Control
0.1 mg
0.5 mg

D.


4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
0.1 mg
0.5 mg

E.

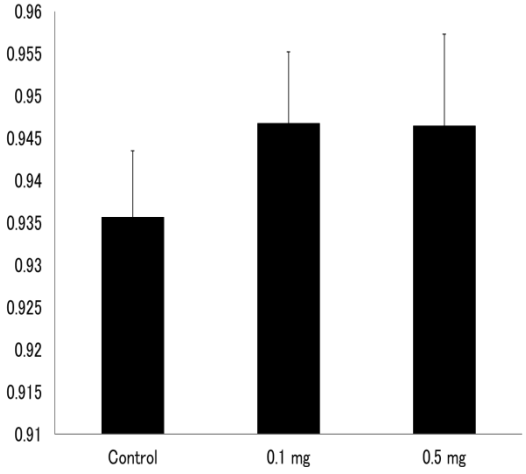

0.96
0.955
0.95
0.945
0.94
0.935
0.93
0.925
0.92
0.915
0.91
Control
0.1 mg
0.5 mg

Figure 4.5 Effects of T-2 toxin on hemodynamic parameters recorded from the common carotid artery in rats at 48 h following subcutaneous administration of the toxin.
A: End-Diastolic Velocity (EDV), B: Peak Systolic Velocity (PSV), C: Mean Velocity (MV), D: Pulsatility Index (PI), E: Resistance Index (RI). The asterisk shows a significant difference at $p<0.05$ (*) between the corresponding bars. Data are expressed as means $\pm$ SEM.

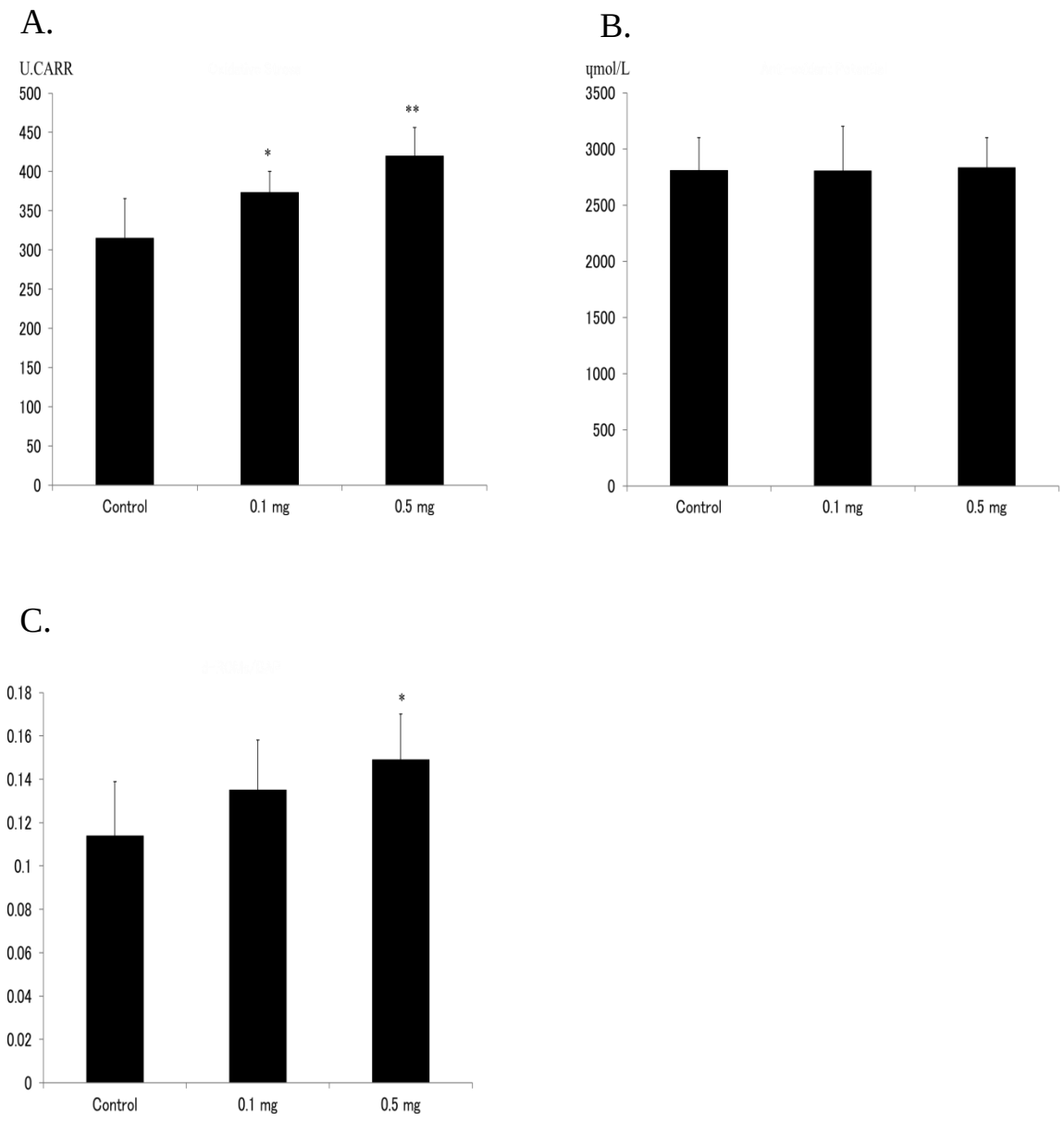


Figure 4.6 Serum at 48 h following vehicle or T-2 toxin subcutaneous administration. A: Oxidative stress level from d-ROMs test, B: Anti-oxidant potential level from BAP test, C: d-ROMs/BAP ratio. The asterisks show significant difference at $p<0.05$ and $p<0.01$ (*,**), respectively from the control group . Data are expressed as means $\pm$ SD.

CHAPTER 5

TOXIC EFFECTS OF T-2 TOXIN AND DEOXYBIVALENOL ON THE MITOCHONDRIAL ELECTRON TRANSPORT SYSTEM OF CARDIOMYOCYTES

5.1 Introduction

In the previous chapters, all the studies were performed by *in vivo* methods and the results showed that DON and T-2 toxin have potent cardiac toxicities. The author considers that cellular levels of experiments are required since a part of those results are suggested to be derived from direct actions on the cardiovascular system. Though the findings that T-2 toxin and DON impaired cardiovascular function and could induce arrhythmias were obtained in chapter 2 and 3, the sites that these toxins affect have not been clarified yet. In chapter 3, it was recognized that the imbalance of ANS activity might be associated with the cardiac dysfunction induced by trichothecene mycotoxins. On the other hand, some cardiac abnormalities such as ventricular extrasystoles were not abolished by the administration of autonomic nervous blockades, therefore a part of arrhythmia induced by T-2 toxin and DON may be caused via their direct effects on cardiomyocytes. However, the evidence of direct toxicity of these toxins on cardiomyocytes at the mitochondria level has not been fully explored.

It has been recognized that mitochondria is most important intracellular organ of which damage can provoke various metabolic dysfunctions and cellular necrosis or apoptosis. Because the mitochondria makes up at least 20% of the cardiomyocyte volume and provides the unremitting energy required for contraction in the heart (David, *et al*., 1979; Schaper, *et al.*, 1985), being the major site of ATP production for tissue survival and functionality. In past reports some studies described the toxic effect of T-2 toxicity on mitochondria, where it inhibited oxygen consumption resulted from inhibition at site I of the electron transport chain (ETS) in the rat's liver (Pace, 1983) or inhibited succinate dehydrogenase activity and site II of ETS in *S. cerevisiae*

(Koshinsky *et al.*, 1988). My interest is that since T-2 toxin has detrimental effect to the mitochondria in other cell types the similar effect would happen to the cardiomyocyte, which is possible to be one of the factors that induces cardiac abnormalities in the previous studies (Feuerstein, *et al.*, 1985). Moreover, DON which is one of trichothecence mycotoxin group members may disrupt mitochondria function in the similar way. Therefore, the hypothesis in the present study is that the trichothecene mycotoxin group interrupts the maintenance of mitochondrial function and the availability of a bioenergetics reserve capacity.

To evaluate mitochondrial function, I used an emerging technology, i.e., the high-throughput extracellular flux (XF) analysis, to qualify the bioenergetics changes that occur in intact cardiocytes exposed to trichothecene mycotoxins. By measuring XF, I was able to measure oxygen consumption rate (OCR) as shown in Figure 5.1. The theoretical idea is that, when the ability of ETS and the bioenergetics reserve capacity is decreased or depleted, cellular injury occurs accompanied by decreased mitochondrial oxygen consumption. Using inhibiting agents (i.e. oligomycin, rotenone, antimycin A) to the ETS allows us to know which part of ETS is affected by trichothecene mycotoxins (Figure 5.3).

Therefore, it should be a key experiment to clarify the change in mitochondrial function by application of mycotoxins. The present study in this chapter focused on the mitochondrial function in cardiomycytes that were exposed to DON and T-2 toxin with wide range of concentrations.

In the present study, the OCR was chosen as an indicator of cellular influences by the toxins because the disorder of respiratory function in cells will lead to the disruption of overall functions in the living body, which results in severe diseases or

even death. From a point of this view, it is natural to consider that the cardiac toxicity such as arrhythmias due to T-2 toxin and DON are partly derived from the cellular damage if some abnormal changes in OCR are present.

The aim of this study is to investigate the toxicity of T-2 toxin and DON against bioenergetics function in mitochondria by measuring OCR.

5.2 Materials and Methods

Materials

T-2 toxin standard (5 mg, 97% purity), DON standard (5 mg, 98% purity) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Carbonyl cyanide-*p*-trifluoromethoxyohenylhydrazone (FCCP, 10 mg, BML-CM120 with 98% purity) was purchased from Cosmo Bio Co Ltd. (Tokyo, Japan). Oligomycin (5 mg) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dimethyl Sulfoxide (DMSO, 500 ml, 99% purity) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Phosphate buffered saline (PBS, 500 ml, pH 7.4) and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F-12) were purchased from Life Technology Japan Ltd. (Tokyo, Japan). Collagen Type I Rat tail (100 mg) was purchased from BD Biosciences (Bedford, MA, USA). All materials and reagents for the XF assays were provided by Seahorse Bioscience Inc. (N. Billerica, MA, USA). T-2 toxin and DON were dissolved in DMSO in order to obtain 5 mg/ml as a stocking solution.

Cell culture

The primary cardiomyocytes that were isolated from neonatal Sparague Dawley (SD) strain rats (1-4 days of age), were purchased from Primary Cell Co., Ltd. (Sapporo, Japan). These cells were seeded at the specified number as described below on to collagen-coated Seahorse Bioscience XF 96 cell culture microplates with 96 wells in growth medium containing 15% fetal bovine serum and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 ℃. The medium was replaced with fresh media every 24 h.

First, the optimum number of cells and the optimum culture time to be used for the experiments on T-2 toxin and DON effects were determined with preliminary tests. For this purpose, cardiomyocytes were seeded at a density of 5000, 10000, 20000, 40000 or 80000 cells /well and cultured for 48 or 72 h. As a result, a seeding density of 20000 cells/well resulted in the minimum cell number that started beating at 48 h and contained 80% confluence, and the culture time of 72 h was chosen for the remainder of the experiments because their most stable OCR responses to both toxins and stress tests by oligomycin and FCCP were obtained.

Incubation of isolated cardiomycocytes with trichothecene mycotoxins

As for the exposure to each toxin, the supernatant was replaced with medium containing T-2 toxin at concentrations of 6×10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, or 10^{-1} μM and DON at concentrations of 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, or 100 μM. The final concentration of DMSO that was used for dissolution of both toxins and the concentration of DMSO in control solution without toxins did not exceed 0.2% (v/v): this concentration of DMSO by itself produced no observable effect on OCR.

Bioenergetic measurements

The procedure was performed with reference to the method of Hill et al., (2009). An XF96 Analyzer (Seahorse Bioscience) was used to measure bioenergetic function in intact cardiomyocytes. For all bioenergetics measurements, the culture medium was changed 1 h prior to the assay run to unbuffered DMEM (pH 7.4, Gibco). In order to further analyze the changes in mitochondrial function due to both toxins, the "stress test" was conducted with oligomycin, which is an inhibitor of complex V (ATPase), and FCCP, a proton ionophore that activates ETS in the mitochondria. After the medium with T-2 toxin or DON was applied for 24 h, oligomycin and FCCP were injected sequentially through ports in the Seahorse Flux Pak cartridges to final concentrations of 3 μM and 2.5 μM respectively. These treatments allowed the determination of the basal level of oxygen consumption, the amount of oxygen consumption linked to ATP production on oligomycin-injection, and reserve capacity (RC) on FCCP-injection. The basal OCR measurements were recorded at 3 consecutive points prior to the injection of oligomycin. After recording the oligomycin-insensitive OCR at consecutive 3 or 4 points, FCCP was injected, and another OCR measurement was recorded. The OCR measurement after the FCCP injection represented the maximal capacity with which the cells could reduce oxygen under the experimental conditions. In the present study "ATP-linked OCR" was defined as the difference in OCR immediately before and after the oligomycin injetion and RC was the difference between the maximum OCR response immediately after the FCCP injection and the basal OCR immediately before oligomycin injection. The theoretical model was referenced to the reports by Hill, et al. (2009) and Dranka, et al. (2011).

Statistical analysis

All results are expressed as the means and standard deviations of data obtained from 6 wells with different concentration of T-2 toxin, DON. For the data within a group, the concentration-response relationship was statistically tested by one-way analysis of variance (one-way ANOVA) and the differences of values between the control and the given concentration of T-2 toxin and DON were evaluated by Dunnett test. *p*-values less than 0.05 were considered statistically significant.

All experiments were conducted in accordance with the Animal Experimentation Guidelines of the University of Tokyo.

5.3 Results

Effects of T-2 toxin and DON on basal OCR

T-2 toxin at concentrations of 6×10^{-8} to 6×10^{-1} μM and DON at concentrations of 0.39 to 100 μM were applied to whole cardiomyocytes for 24 h after 72 h-culture without toxins. The basal OCR decreased to 71.9 % at 6×10^{-5} μM of T-2 toxin compared to the control and the extent of the decrease was the maximum with 6×10^{-4} μM to 6×10^{-1}μM, which was 15.4 % to 18 % (Figure 5.3A and 5.4A) of the control. Likewise, the basal OCR decreased to 81 % at 0.78 μM and the lowest OCR, 25.9 % to 39.8 %, was observed with 6.25 to 50 μM on DON compared to the control (Figure 5.3B and 5.4B). A significant concentration-dependent manner on the basal OCR was observed with both mycotoxins ($p<0.001$, One-way ANOVA). In these experiments DMSO alone caused no significant effects on OCR.

ATP-linked OCR and reserve capacity in mitochondria

After the measurement of basal oxygen consumption in cardiomyocytes that were exposed to T-2 toxin or DON for 24 h, oligomycin and FCCP at a final concentration of 3 μM and 2.5 μM, respectively, were injected into the culture wells. As shown in Figure 5.4, oligomycin decreased the OCR, and, in contrast, FCCP increased it in both the control and toxin groups in a concentration-dependent manner ($p<0.001$, One-way ANOVA).

The ATP-linked OCR significantly decreased in a concentration-dependent manner ($p<0.001$, One-way ANOVA). The ATP-linked OCR was significantly decreased with $6x10^{-5}$ μM ($p<0.05$) or higher concentration ($p<0.0001$) in T-2 toxin groups compared to the control (Figure 5.5A). A significant decrease in ATP-linked OCR was also observed with 0.78 μM ($p<0.001$) or higher concentrations ($p<0.00001$) (Figure 5.5B) in the DON groups.

The mitochondrial RC was reduced in both T-2 and DON-groups in a concentration-dependent manner ($p<0.001$, one-way ANOVA). T-2 toxin resulted in a significant reduction of RC at a concentration of 6×10^{-5} ($p<0.005$) or higher ($p<0.0001$) (Figure 5.6A), while DON resulted in also significant RC reduction at 3.13 μM ($p<0.05$) or higher ($p<0.001$ to 0.005) except for 6.25μM ($p>0.05$) (Figure 5.6B).

5.4 Discussion

In the present study we evaluated the effects of the representative mycotoxins that belong to the tricothecene group on the bioenergetics function in intact cardiomyocyte with an extracellular flux analyzer. The findings from the OCR measurements elucidated that the cardiomyocytes exposed to T-2 toxin and DON for 24

h exhibited a reduced basal OCR. This finding is not likely to have been derived from a decrease in the number of live cardiomyocytes because the OCR that was corrected for the number of surviving cardiomyocytes also showed a significant dose-dependent reduction of OCR with T-2 toxin in our preliminary study. Therefore, these toxins were assumed to have the property of cellular toxicity due to mitochondrial dysfunctions. Indeed, the OCR changes in response to oligomycin and FCCP demonstrated the reduced function of ETS in mitochondria as compared to the controls which were exposed to medium without toxins.

In accordance with the results obtained from the OCR changes in response to olygomycin and FCCP, the threshold of cardiomyocyte toxicity was estimated to be between 6.0×10^{-6} and 6.0×10^{-5}μM for T-2 toxin and between 0.39 and 0.78μM on ATP-linked OCR or between 1.56 and 3.13μM on RC for DON in the present study. Therefore, the OCR-toxicity of T-2 toxin on cardiomyocyte was conceived to be approximately 13000 times higher than that of DON if the comparison was made between 6.0×10^{-5}μM (T-2 toxin) and 0.78μM (DON).

Mitochondria play a critical role in mediating the cellular responses to oxidants that are formed during acute and chronic cardiac dysfunction. It is widely assumed that, as cells are subjected to stress, mitochondria are capable of drawing upon a RC that is available to serve the increased energy demands for the maintenance of organ function, cellular repair or the detoxification of reactive species. The impairment or depletion of this RC might lead to excessive protein damage and cell death. As mentioned above, the result from the present study with FCCP showed a marked inhibition of the RC of the ETS in the mitochondria with a wide range of concentrations (3.13 to 100 μM) of DON. Recently, it was demonstrated that DON at 100 μM triggers a mitochondria-dependent

apoptosis in human colon carcinoma cells (HCT116) due to opening of permeability transition pore, the generation of super oxide anion, and cytochrome c release that is associated with caspases activation (Bensassi, F. et al., 2012). Similarly, Ma Y. et al. (2012) demonstrated that DON-induced apoptosis in human colon cancer cells (HT-29) was caused by mitochondrial dysfunction, and the subsequent release of cytochrome c into the cytoplasm, and the successive activation of caspases in addition to the morphological changes that were represented by swelling, the absence of crista, and the vacuolization of mitochondria. In accordance, the DON-induced reduction of mitochondrial RC of cardiomyocytes in the present study may be associated with apoptosis pathway in the mitochondria although the cultured cells were different from each other.

Mitochondrial dysfunction or genetic abnormalities lead to hypertrophic cardiopathy, arrhythmias and sudden cardiac death (Marin and Goldenthal, 2002; Di, D.S., 2009). In addition, cardiac mitochondria are considered a necessary organelle that is involved with cardiomyocyte excitability. The fluctuation of sarcolemmal K_{ATP} currents, and consequently, the action potential duration in myocardium are intricately linked to the behavior of the mitochondria, where the relationship between the collapse of the mitochondrial membrane potential and the sarcolemmal K_{ATP} in the myocardium subjected to oxidative stress has been described (Brown and O'Rourke, 2010). Such findings of mitochondrial dysfunction may be concerned with generation of arrhythmias such as ventricular extrasystoles that were recognized in the previous chapter on DON and T-2 toxin. It is possible that the mitochondrial dysfunction caused by these toxins is largely due to oxidative stress. A recent study (Fang, H. et al., 2012) showing that the T-2 toxin-induced cell damage and apoptosis were caused by reactive oxygen

species-mediated mitochondrial pathway also confirms such a possibility.

The present study clarified the reduced activity of ETS linked to ATP-production and also the reserve capacity in ETS caused by both T-2 toxin and DON. It is reasonable to consider, therefore, that such mitochondrial dysfunction produces bioenergetics imbalance between oxygen supply and oxygen demand (ATP production) in the exposure to each toxin and that serious cell damages can result in generation of various cardiac abnormalities such as ventricular arrhythmia.

In conclusion, the present study demonstrated that T-2 toxin and DON, representative tricothecene group of mycotoxins, have a potential leading to mitochondrial dysfunction of cardiac muscles, which might induce various cardiac disorders.

5.5 Summary

In order to study direct effects of T-2 toxin and DON on the heart tissue, cultured myocytes isolated from newborn rats were assigned for experiments to measure the oxygen consumption rate (OCR) when T-2 toxin and DON were applied to the myocytes. T-2 toxin and DON markedly inhibited the bioenergetics function in the mitochondrial electron transport system, where the basal OCR and ATP-linked OCR in response to oligomycin and the reserve capacity in response to FCCP were significantly decreased by both toxins. Such changes in the mitochondrial function was observed at a low concentration of T-2 toxin as low as 6×10^{-5}μM, whereas the higher concentration, 0.78μM, of DON produced the mitochondrial dysfunction.

These findings demonstrated that the secure cytotoxicity to the cardiac cell was present in T-2 toxin and suggested that some cardiac dysfunction by T-2 toxin can be

provoked by the direct action of this toxin.

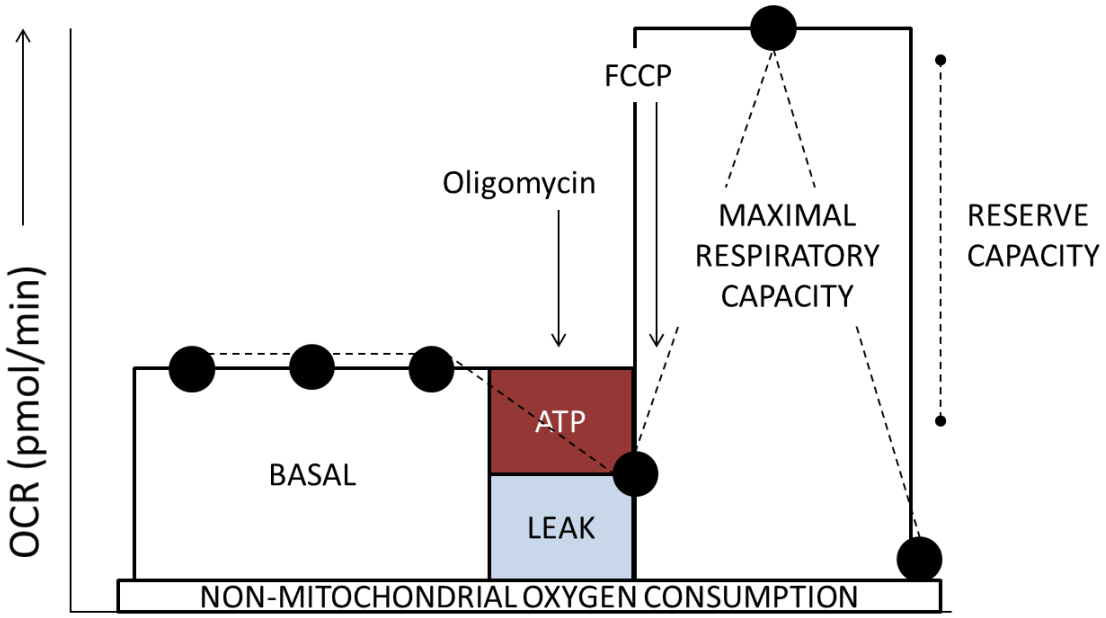


Figure 5.1 Measurement of bioenergetics parameters in cardiocytes using XF technology (Hill et al., 2009)

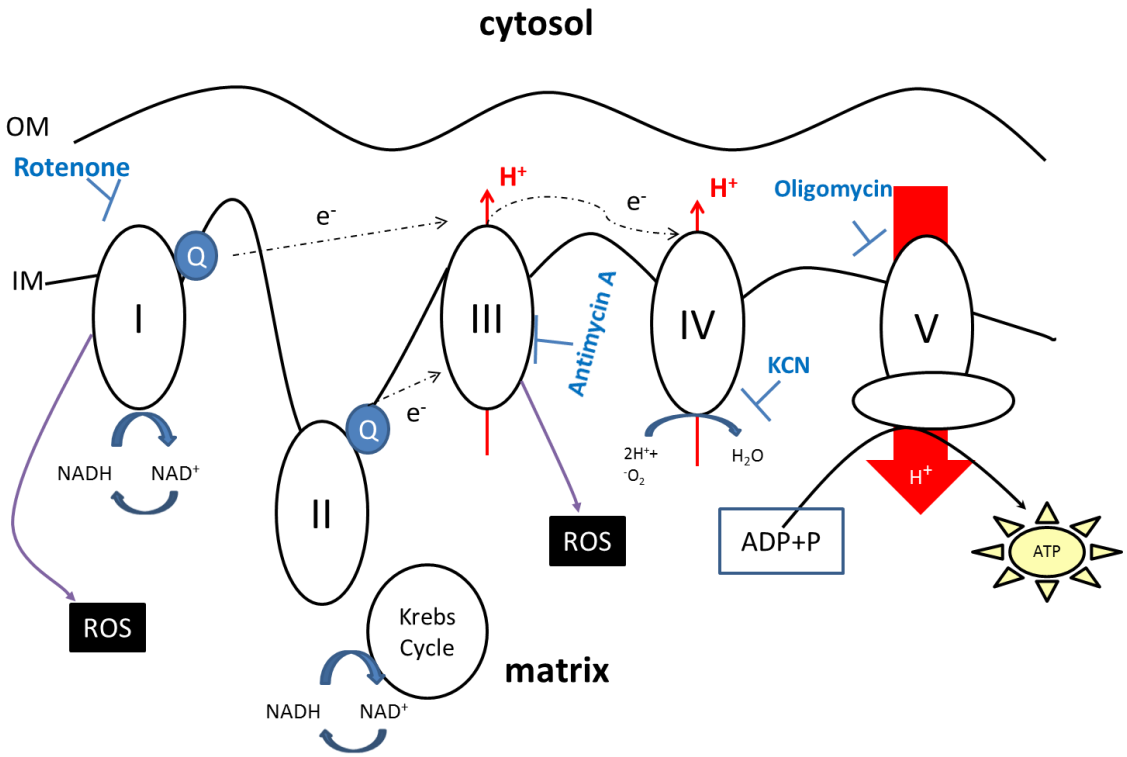
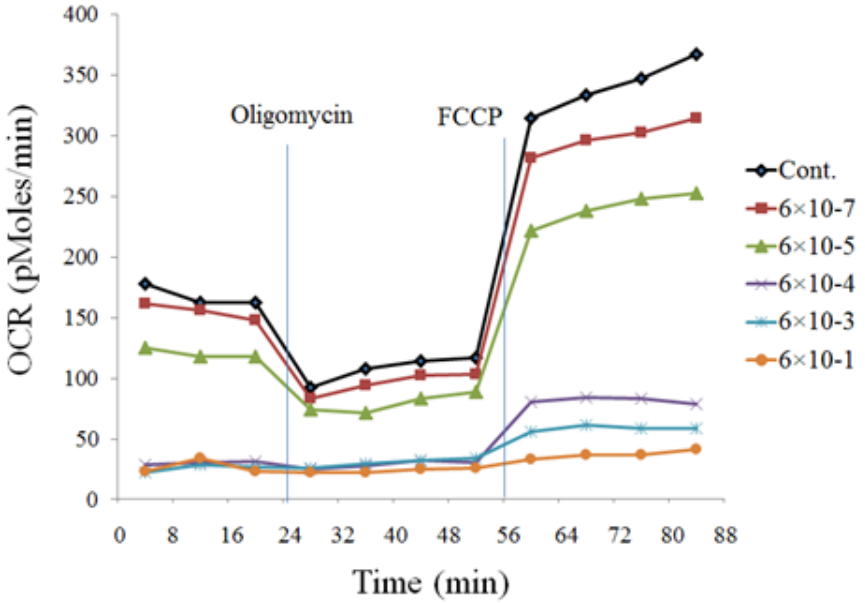


Figure 5.2 Electron transport chain, ATP generation and ROS production. Different complexes (I-V) of the electron transport chain are represented in the inner membrane (IM) of the mitochondria. Several respiratoy inhibitors are represented: rotenone: complex I inhibitor; antimycin A: complex III inhibitor; potassium cyanide (KCN): complex IV inhibitor, and oligomycin: complex V inhibitor (Ricci et al., 2003)

A.



B.

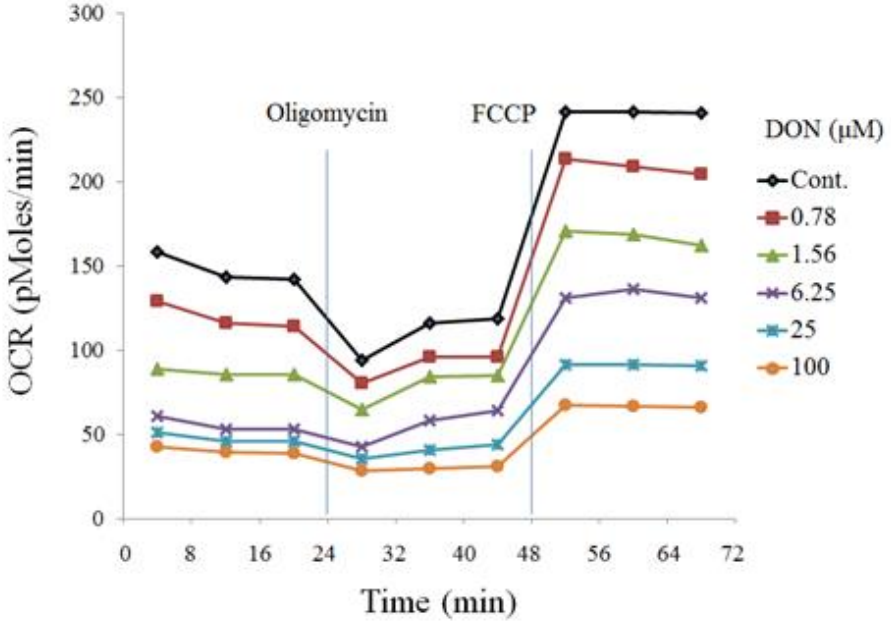
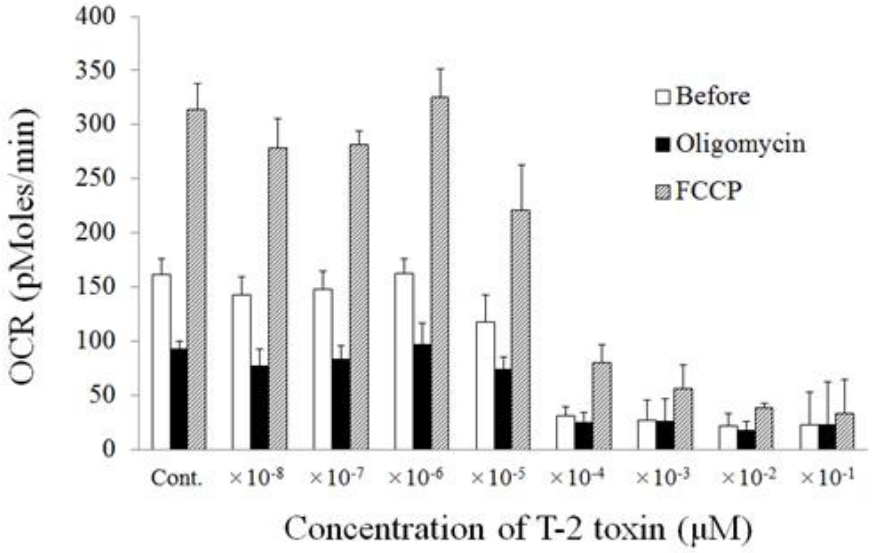


Figure 5.3 An overview of the oxygen consumption rate (OCR) changes in response to the different doses of T-2 toxin (A) and deoxynivalenol (DON) (B) and the effects of oligomycin and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) injections.

After basal OCR was measured, oligomycin (3μM) and FCCP (2.5μM) were injected sequentially during continuous OCR measurements. Not all doses were shown in order to avoid complexity in the figures. Cont: vehicle solution without toxins; OCR: mean oxygen consumption rate from 6 wells. The data are expressed as means ± SD (n=6).

A.



B.

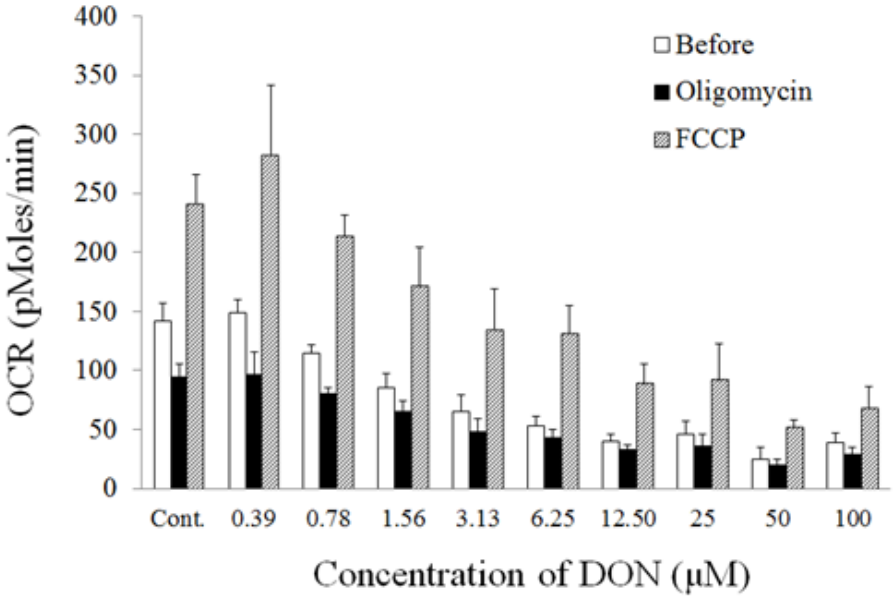
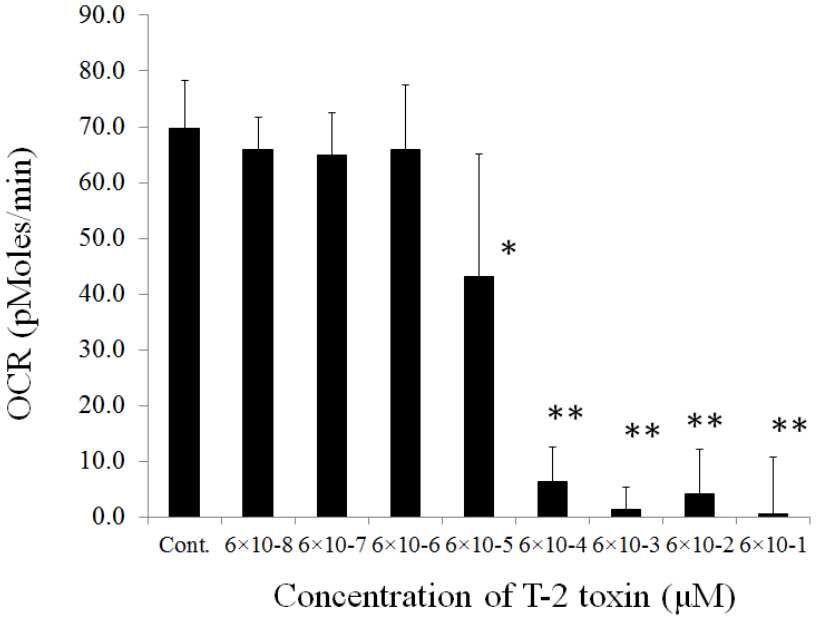


Figure 5.4 Effects of oligomycin and FCCP on OCR in cardiomyocytes exposed to T-2 toxin (A) and DON (B).

Before: immediately before oligomycin-application; Oligomycin: immediately after oligomycin-application; FCCP: immediately after FCCP-application; Cont: vehicle solution without toxins; OCR: mean oxygen consumption rate from 6 wells. The data are expressed as means $\pm$ SD (n=6). In A, the concentration is expressed as $6x10^{-8}$-10^{-1}μM.

A.



B.

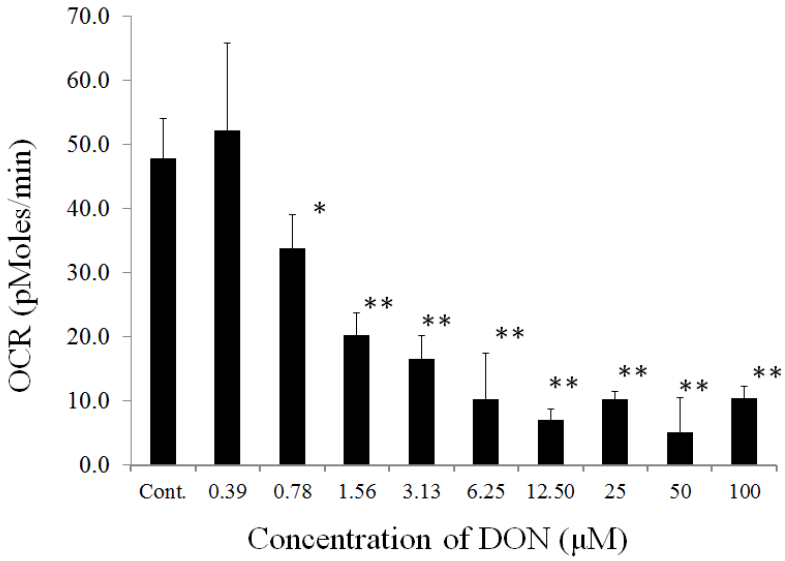
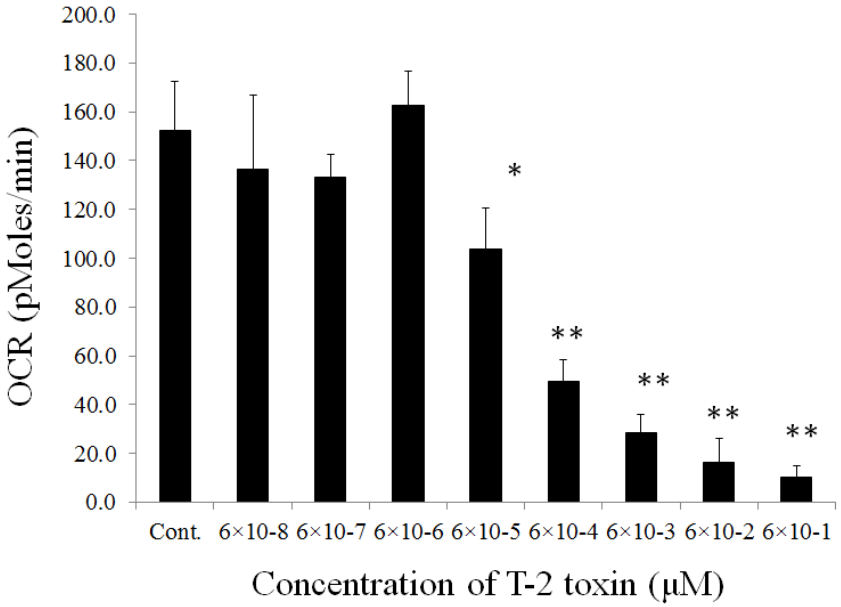


Figure 5.5 ATP-linked OCR estimated by oligomycin-induced OCR changes in cardiomyocytes exposed to T-2 toxin (A) and DON (B).

Significant differences compared to the control are shown. In A, the concentrations of the horizontal axis are expressed as $6x10^{-8}$-10^{-1}μM. Cont: vehicle solution without toxins; OCR: mean oxygen consumption rate from 6 wells. (A) *: $p<0.05$ vs Control, **: $p<0.0001$ vs Control. (B)*:$p<0.001$ vs Control, **:$p<00001$

The data are expressed as means ± SD (n=6).

A.



B.

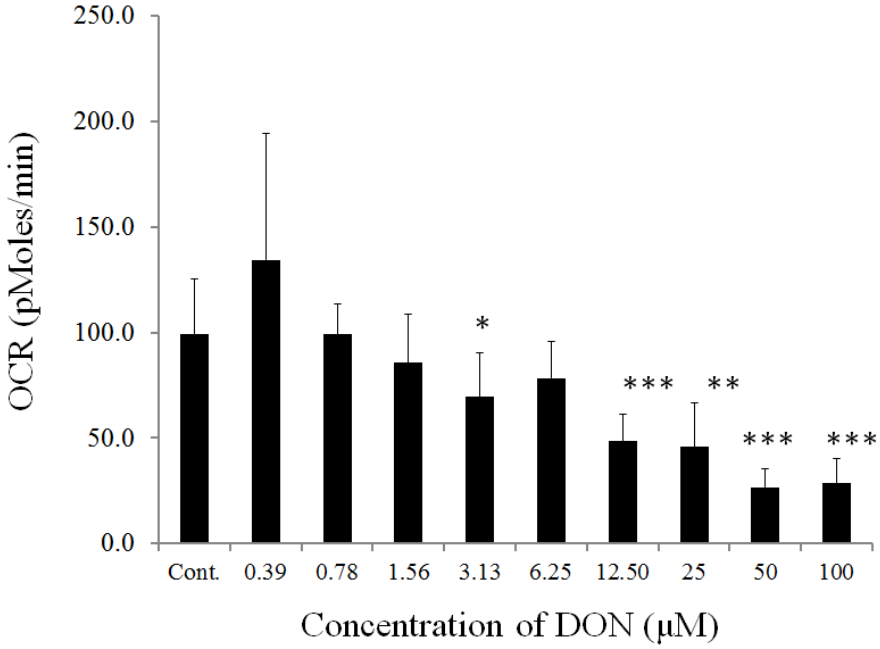


Figure 5.6 Reserve capacity estimated by FCCP-induced OCR changes in cardiomyocytes exposed to T-2 toxin (A) and DON (B).

Significant differences compared to the control are shown. In A, the concentrations of the horizontal axis are expressed as 6x10-8-10-1μM. Cont: vehicle solution without toxins; OCR: mean oxygen consumption rate from 6 wells. (A)*: $p<0.005$ vs Control, **: $p<0.0001$ vs Control. (B)*:$p<0.05$ vs Control, **:$p<005$ vs Control, ***:$p<0.001$ vs Control. The data are expressed as means $\pm$ SD (n=6).

CHAPTER 6

GENERAL DISCUSSION

Trichothecene mycotoxin is a toxic secondary metabolite from *Fusarium spp*. which is the fungi that can grow well in a hot and warm climate country like Japan (Tanaka et al., 2010). Though there were several reports about toxicity of T-2 toxin to the cardiovascular system (Feuerstein et al., 1985, Bubien & Woods, 1986, 1987, Yarom et al., 1986, Magnuson et al., 1987), there are still limited data about DON toxicity. Furthermore, until the present, most of the toxicological study focused on acute effects of DON and T-2 toxin after toxin administrations in a few hours and detail investigations on the cardiovascular effects including the relationship of the autonomic nervous activity that induces cardiovascular dysfunction has not been clarified yet.

Therefore, the aim of this study was to investigate cardiovascular and autonomic nervous responses to these trichothecene mycotoxin exposures and attempt to elucidate mechanisms and targets of this mycotoxin in causing dysfunction in acute and long-term observation.

In chapter 2, the acute effects that occurred within 3 h following subcutaneous injection of DON or T-2 toxin under anesthetic condition were examined. The administration of both DON and T-2 toxin immediately developed an increase in MBP. A decrease in HR was observed in DON administration while T-2 toxin caused an increase of HR at the same period of the observation. Changes in ECG-waveform with the sporadic arrhythmias were induced by both DON and T-2 toxin administration. ECG and BP findings from the measurements in this chapter demonstrated that DON and T-2 toxin has a potential to produce the cardiovascular alteration.

In chapter 3, the changes in cardiac and autonomic nervous activity following administration of DON and T-2 toxin were observed for longer period in unrestrained and conscious rats. According to the long-term and continuous observation, it was

elucidated that both the toxins induced cardiac abnormalities including various types of arrhythmia. There was a difference in the toxic intensity between DON and T-2 toxin. DON has less toxic effect having cardiac abnormalities mostly decayed within 6 h, whereas T-2 toxin has the stronger effects in which cardiac abnormalities lasted for as long as 72 h following the subcutaneous administration. The frequent appearance of arrhythmia accompanied by an increased in HR and the prolongation of QRS duration and QT interval during 3 days was observed following T-2 toxin administration, while DON almost lost its toxicity within a day of injection. Furthermore, the power spectrum, a parameter of autonomic nervous activity, showed a marked alteration which suggests that the entire outflow of the autonomic nervous system was inhibited in rats administered a high dose of the toxin.

Arrhythmias that were identified in chapter 3 predominantly appeared after 8 and 10-20 h after T-2 toxin and DON administration, respectively. It is noteworthy to recognize that no obvious arrhythmias were observed immediately after these toxins injection. The relationship between the occurrence of cardiac abnormalities and the autonomic nervous activity that was observed after the administration of DON and T-2 toxin was unclear. Therefore, the responses to the T-2 toxin from the autonomic nervous activity of which cardiac toxicity was less than in DON were investigated in the absence of regulation. The rats that received continuous infiltration of propranolol preceding administration of T-2 toxin failed to show the increase in HR by T-2 toxin. This finding suggests that the increase in HR that was induced by T-2 toxin was influenced by an augmented activity in the sympathetic nervous system. In addition, the pretreatment with atropine and propranolol attenuated the occurrence of arrhythmias in this study. According to the results, it was demonstrated that T-2 toxin affected the autonomic

nervous activity especially sympathetic limb which brought to an increase of HR. Such alteration of the autonomic nervous activity might be thought to be mediated by the baroreflex which is accelerated by hypotension. In fact, the tendency for decrease in the peripheral vascular resistance by T-2 toxin was found, being described in chapter 4.

In chapter 4, the circulatory function at 48 h following T-2 toxin administration was observed by using echocardiogram. Furthermore, the oxidative stress induced by T-2 toxin was measured in serum. The higher dose of T-2 toxin increased the HR, SV and CO. The increase in CO has been thought to be an important physiological function to protect the body from severe hypotensive condition. Furthermore, the tendency for increase of PSV and MV in the common carotid artery was also observed in this study. These findings are consistent with the evidence for the increase in CO after the high dose of administration with T-2 toxin.

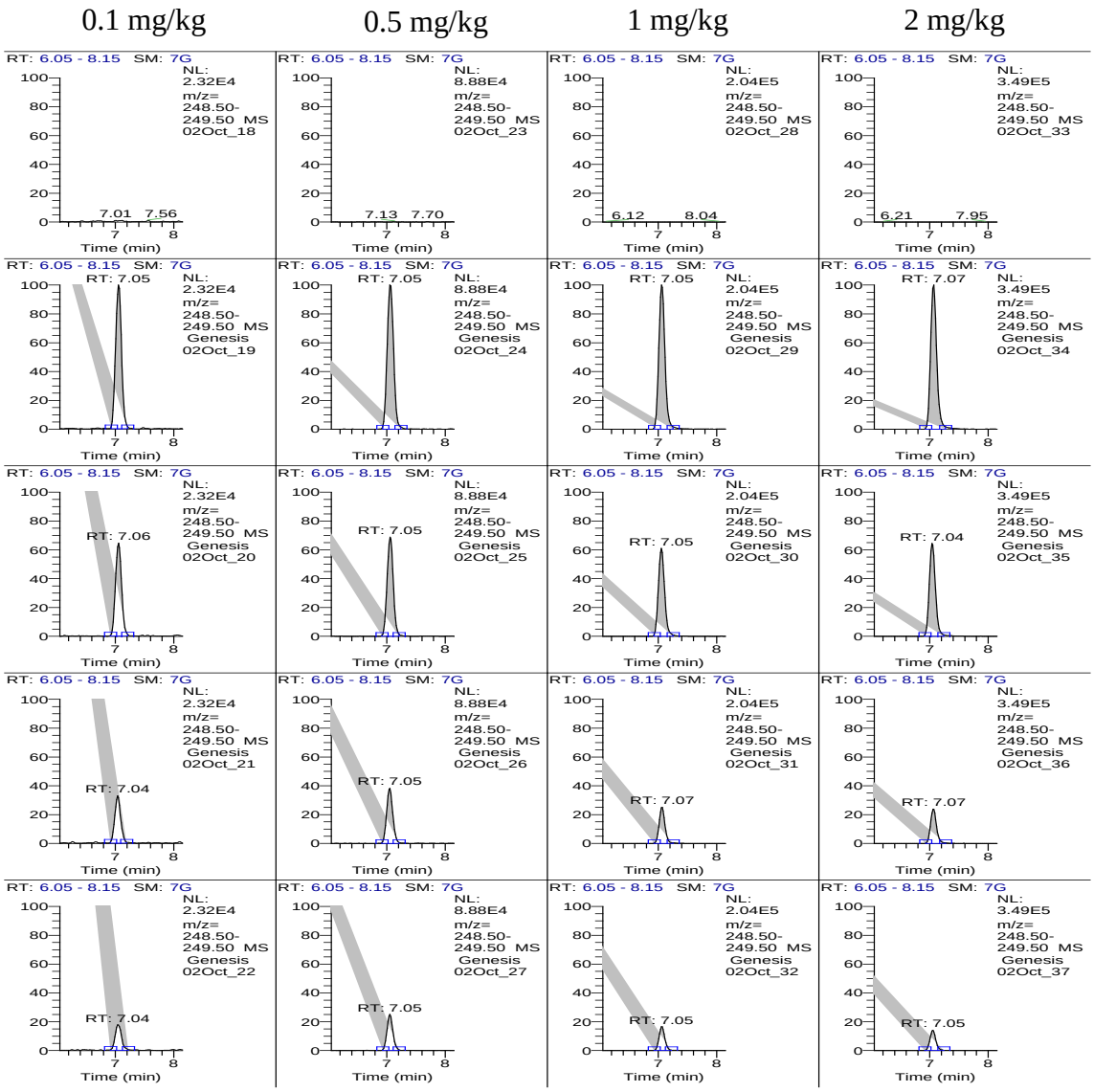
The oxidative stress that was evaluated by measuring ROS production in T-2 toxin treated-group was significantly higher than the control group with a dose-dependent manner. However, the anti-oxidant potential was not increased when compared with the control. Therefore, the balance (d-ROMs/BAP ratio) between oxidative stress and anti-oxidative stress exhibited the marked increase in the oxidative stress. It is considerable that oxidative stress might be one of the underlying mechanism by which T-2 toxin causes the cardiovascular dysfunction in the present study.

In chapter 5, the *in vitro* effect of DON and T-2 toxin on cardiomyocytes was investigated through measurement of OCR which reflects the function of electron transport system (ETS) in mitochondria. This experiment evidenced that a considerable dysfunction of ETS in which a marked extent of reduction of ATP production-linked ability was provoked. In past studies many reports have described that mitochondrial

dysfunction causes the abnormalities in the cardiovascular system (Brown and O'Rourke, 2010 and Ke et al., 2012). The present study attempted to find a possible assumption between OCR changes induced by both the toxins and cardiac dysfunctions such as arrhythmia. The findings from the OCR measurements elucidated that the cardiomyocytes exposed to T-2 toxin and DON for 24 h exhibited a reduced basal OCR with a decline in activity of ETS linked to ATP-production and also the reserve capacity in ETS. Therefore, these toxins were considered to have the property of cellular toxicity due to mitochondrial dysfunction that can result in generation of various cardiac abnormalities such as ventricular arrhythmias. Although the direct demonstration was not performed in the present study, the presence of causal relationships between the cellular damage in the heart and the generation of arrhythmias is considered sufficient.

In conclusion, the present study, including *in vivo* and *in vitro* experiments, elucidated that both DON and T-2 toxin which are representative trichothecene type of mycotoxin have a property to produce cardiovascular abnormalities of which mechanisms are associated with abnormal function of the autonomic nervous system and cellular dysfunctions in the myocyte. These findings are thought to provide useful data as measurement for food and feed safety in humans and livestock animals.

Additional Data
(DON plasma concentration)



DON concentration reached peak in the plasma at 30 min following subcutaneous administration in every dose of injection. 0 min is the time before DON administration and served as a control.

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