Studies on stress by heat and transport in swine.

豚の暑熱・輸送ストレスに関する研究

Doctoral Dissertation

Joint Graduate School of Veterinary Science, Kagoshima University

Moe IJIRI

2024

TABLE OF CONTENTS

ABBREVIATIONS

1,5-AF: 1,5-Anhydro-d-fructose

1,5-AG: 1,5-Anhydro-d-glucitol

APPs: acute phase proteins

Alb: albumin

A/G ratio: albumin/globulin ratio

BAP: biological antioxidant potential

CK: creatinine kinase

d-ROMs: derivatives of reactive oxygen metabolites

Glb: globulin

HPA axis: hypothalamic-pituitary-adrenal axis

LDH: lactate dehydrogenase

NEFA: non-esterified fatty acids

PSE meat: pale, soft and exudative meat

ROS: reactive oxygen species

SAA: serum amyloid A

SA system: sympathetic adreno-medullary system

SNS: sympathetic nervous system

TP: total protein

GENERAL INTRODUCTION

Pigs were among the first animals, along with goats, sheep, and cattle, to be domesticated in the transition to a sedentary society approximately 10,000 years ago (1). Pigs were domesticated from wild boars (*Sus scrofa*) and various traits, including growth rate, litter, and body size were altered to make them more productive. Commercial breeds, especially in Europe, have long been selectively bred for body elongation to increase meat production and improve reproductive performance (2-4). The efficient meat production through intensive production systems is becoming more important with a background of the increase in global demand for livestock products.

Meanwhile, the demand for pigs as experimental animals has also increased in recent years. Swine are considered to be increasingly attractive toxicological and pharmacological models because of their well-accepted physiological and anatomical similarities to humans (5-10). Additionally, the ethical issue associated with using dogs and monkeys as experimental animals have increased their usefulness (11). In contrast to stockbreeding, smaller body size is expected in experimental animals because of their ease of rearing and handling (12). Experimental minipigs, defined as smaller than domestic pigs and weighting less than 100 kg, have been developed including the strains of Göttingen, Yucatan, Sinclair, Clawn, and Microminipigs (8).

Pigs can be expected to be exposed to variety of stresses in the process of their rearing in both meat production and experimental use. In livestock industry, it is common to apply the intensive systems, which have been believed to produce stress in animals (13). For example, weaning, regrouping within the productive cycle, and transportation to slaughterhouses or other rearing areas are very common processes in modern swine farming, but these processes have been reported to cause stress in pigs (13). In addition, inappropriate husbandry management, including food and/or water restrictions (14-16), inappropriate environmental conditions for farming (17) and exposure to pathogenic factors (18), can also cause stress. There has been growing concern regarding stress among pigs in recent decades because of the undesirable consequences that stress imposes on the normal physiology of pigs, their welfare, and general productive performance.

In modern intensive pig production, the temperature in the pig house is generally adjusted to appropriate conditions for pigs at each production stage. However, sometimes optimal environmental conditions cannot be maintained in farms producing stress in animal (13). Heat stress has an adverse impact on livestock production as elevation of temperature can result in compensatory changes in animal behavior, physiology, and metabolism. This impact is particularly marked in pigs as they are more poorly adapted to heat dissipation than other livestock species because of the paucity of sweat glands and the thick subcutaneous fat. The porcine response to increasing environmental temperatures appears to be based on reducing metabolic heat production to maintain euthermia (19). One example of a behavioral response in pigs is the reduction of metabolic heat by decreasing feed intake, which is effective because digestion and nutrient absorption generate heat (20). Physiologically, elevations in environmental temperature also directly alter nutrient utilization, energy metabolism, and hormonal regulation (21-23). Consequently, heat stress can cause substantial economic losses for the swine industry through its effect on growth rates, carcass composition, sow performance, mortality and morbidity (24-26).

Despite the fact that transportation is an unavoidable production process in the swine industry, it is well known that pigs being transported are exposed to various stressful events including departure from the usual room, truck loading and unloading, fasting, different temperature and humidity, noise, vibration of the vehicle or inappropriate stocking densities (27, 28). During transport pigs exhibit physical changes such as weight loss, increased circulating concentrations of catecholamines, cortisol and creatine phosphokinase, increased heart rate and packed cell volume, and dehydration (28). Moreover, when inadequate transport conditions are combined with a genetic mutation of pig that carry the recessive ryanodine receptor type 1 gene, the aforementioned stressful factors can trigger more severe metabolic alterations known as porcine stress syndrome (29, 30). Pigs with porcine stress syndrome are considerably more susceptible to stress and prone to disease, including subclinical disease, as well as to a degradation of meat quality known as PSE (pale, soft and exudative) meat (31, 32). PSE usually occurs in pigs that are genetically sensitive to stress when subjected to acute pre-slaughter stressors immediately prior to slaughter, though it can occur in normal pigs subjected to stressful conditions before slaughter (33). The degradation of meat quality and health damage to pigs caused by transportation stress not only causes economic losses, but also needs to be improved from the perspective of animal welfare.

Kagoshima Prefecture is one of the most prosperous regions for pig farming in Japan, but it is not a favorable region when it comes to the stress factors mentioned above. One of the first is that Kagoshima Prefecture belongs to a subtropical climate zone with high temperatures throughout the year. There is concern that this causes heat stress to the pigs, affecting their health and meat quality. Another is that there are many remote islands where slaughterhouses and other facilities are inadequately developed, and pigs are being raised even in such areas. For example, 'Amami-Shimabuta' (indigenous middle black pig breed) in the Amami-Oshima Island, located approximately 400km south of mainland Kagoshima Prefecture in southwest Japan, is raised as a regional culinary specialty. However, until recently, there was no well-equipped slaughterhouse on the island, and the similar situation is found in some other islands. Thus, it was necessary to transport live animals by long voyage to the appropriate facility if a certain level of hygiene standards were required for marketing. Although this potential productivity loss due to various stress exposures has been suggested, few studies have been conducted on the actual situation.

Although there are various definitions of stress, Moberg, GP (34) defined it as "a response to stimuli that violate homeostasis," which is the most widely known and applicable to both humans and animals. The manifestations of stress are commonly linked to enhanced activity of the hypothalamic-pituitary-adrenal (HPA) axis and the activation of the sympathetic adreno-medullary (SA) system (35). The main methods used to assess stress are direct behavioral observation and the use of biomarkers that reflect physiological responses (13). Stress assessment is very useful in evaluating the achievement and effectiveness of animal welfare. However, there are many factors that affect stress and their complex interactions, and intervention of researcher and handling for analysis itself can be stressful to pigs. For these reasons, there is no gold standard procedure to determine with accuracy the level of pig stress (13).

In this dissertation, I focused on the effects of stress during swine feeding and management. In Chapter 1, I described the effects of heat stress on the pigs and biomarkers for assessing heat stress. In Chapter 2, I evaluated the effects of transport stress on the pigs and examined the efficacy of the acupuncture treatment as a remedial method.

Research Objectives

In this dissertation, the following objectives were established to reveal the effects of stress on pigs and how to improve these effects.

AIM1. To investigate appropriate biomarkers to assess stress in the actual rearing environment of pigs

AIM2. To evaluate the effects of heat stress in a summer environment.

AIM3. To evaluate the effects of stress during transport of pigs.

AIM4. To investigate ways to reduce stress in pigs.

CHAPTER1

Potential biomarkers for chronic seasonal heat stress in Kagoshima Berkshire pigs reared in the subtropical region

This work has been published as:

Ijiri M, Odo K, Sato M, Kawaguchi M, Fujimoto Y, Miura N, Matsuo T, Hou DX, Yamato O, Tanabe T, Kawaguchi H (2022). Potential biomarkers for chronic seasonal heat stress in Kagoshima Berkshire pigs reared in the subtropical region *Vet Res.,* **66**, 209-214.

1.1 Introduction

Pigs are less adaptable to heat dissipation than other livestock species due to their anatomical characteristics. Therefore, the negative effects of heat stress on productivity are known to be particularly pronounced in pigs. Researchers have proposed a number of potential markers of porcine heat stress, often based on studies of it at elevated temperatures under laboratory conditions. Candidates have been suggested based on the inflammatory and acute-phase response known to be induced by heat in humans and pigs (21, 36). Among the proteins involved in the acute response, serum amyloid A (SAA) and serum albumin (Alb) have been identified as positive and negative acute-phase reactants, respectively (37). Acute phase proteins (APPs) such as SAA, haptoglobin, and C-reactive protein are recognised markers of inflammation and have also been proposed as indicators of stress in cattle (38) and pigs (17).

Recent advances in detecting reactive oxygen species (ROS) using derivatives of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) have opened potential avenues in biomarker research which could be exploited for strategies to combat porcine heat stress. Heat stress is generally considered to trigger oxidative stress in livestock animals (39), and acute heat stress in pigs has been shown to cause oxidative damage and up-regulation of antioxidant enzymes in their oxidative muscle (39). Chronic exposure to high environmental temperatures accelerates ROS generation, potentially inducing cytotoxicity ranging from lipid peroxidation to oxidative damage to protein damage (40). As demonstrably established markers of oxidative stress in areas of bioresearch such as human lifestyle diseases (41), d-ROMs and BAP represent a promising line of investigation as markers of such stress in animals, but have only been evaluated in a limited number of studies in domestic animals so far (42-45).

Field conditions vary with season and climate zone, meaning that research during different seasons in diverse areas of the globe can yield data which complement each other. Kagoshima Prefecture in southwest Japan is a major pig-producing area located in the subtropical zone. The Kagoshima Berkshire pigs raised in this prefecture are used by one of the most famous pork brands in Japan, called 'Kagoshima Kurobuta'. Pigs raised in the subtropical summer are likely to be subject to a milder form of heat stress than those investigated previously in many laboratory-based studies. The subtropical summer represents a chronic form of heat stress, and Kagoshima Prefecture is thus a suitable location to elucidate sensitive markers of chronic heat stress in pigs under field conditions.

Accordingly, in this study, I investigated potential markers of chronic heat stress by comparing Kagoshima Berkshire pigs finished in the summer (a period of chronic heat stress) and in the winter (a thermoneutral period) in Kagoshima Prefecture. The evaluations included assessment of carcass traits (made at time of slaughter), biomarkers previously established for heat stress, as well as d-ROMs and BAP as markers of oxidative stress.

1.2 Materials and Methods

1.2.1 Animals and experiment design

Twenty-four finishing pigs (Kagoshima Berkshire) were reared on commercial pig farms in Kagoshima Prefecture. Twelve pigs were allocated to a summer finishing cohort (August to September with average local temperatures of 29.6°C and 26.4°C, respectively) and 12 pigs were allocated to a winter-finishing cohort (November to December with average local temperatures of 16.0°C and 12.2°C, respectively).

The temperature and relative humidity (%) in the pig housing area were recorded on an hourly basis (Thermo-Hygro Data Logger RX-350TH; AS ONE Corporation, Osaka, Japan) and represented as mean ± SD.

The pigs were slaughtered at the end of the two-month finishing period and whole blood was collected during the pigs' exsanguination. The research was carried out according to the Institutional Guidelines for Animal Experiments, in compliance with the Japanese Act on Welfare and Management of Animals (Act No. 105 and Notification No. 6).

1.2.2 Inspection of carcasses and internal organs disposal

Each slaughtered pig's back fat thickness and carcass weight were measured, and the quality of its carcass was assessed based on these parameters according to the criteria of the Japan Meat Grading Association (under which grade 3 represents high quality, grade 2 represents middle quality, grade 1 low quality, and grade 0 substandard). Organs and tissue were checked as part of the routine meat inspection, and instances of condemned organs were recorded to determine the overall incidence. All these tests were performed blind.

1.2.3 Blood sample analysis

The plasma was obtained by centrifugation (4°C, 3,000 rpm, 15 min) and used for the measurement of catecholamines. The serum was obtained by centrifugation (room temperature, 3000 rpm, 15 min) and used for subsequent measurements. Analyses for biochemical parameters (lactate dehydrogenase, creatinine kinase, total protein, serum albumin, serum sodium and chloride level), cortisol and catecholamine were commissioned to the Clinical Pathology Laboratory, Co. Ltd. (Kagoshima, Japan). The concentration of serum globulin (Glb) and albumin/globulin (A/G) ratio were calculated from the measured values for total protein (TP) and serum Alb.

1.2.4 Measurement of serum amyloid A (SAA) concentration.

The concentration of SAA was measured using a Pentra C200 automated biochemical analyser (HORIBA ABX SAS, Montpellier, France) with an SAA reagent specialised for animal serum or plasma (VET-SAA 'Eiken' Reagent; Eiken Chemical Co., Ltd., Tokyo, Japan). In addition, the concentration of SAA was calculated on the basis of a standard curve made by a VET-SAA Calibrator Set (Eiken Chemical Co., Ltd.).

1.2.5 Measurement of blood oxidative stress

Serum d-ROMs and BAP represent reactive oxygen metabolites and antioxidant capacity, respectively. Serum levels of these two markers were measured using a FREE Carrio Duo Redox Analyzer system (Wismerll, Tokyo, Japan), which included a photometer and a thermostatically regulated mini-centrifuge. Both the d-ROMs and BAP tests were conducted according to the manufacturer's instructions.

1.2.6 Statistical analysis

Numerical data other than the incidences of condemned organs are expressed as mean ± standard deviation. All data were analysed using IBM SPSS Statistics 25 software (IBM, Armonk, NY, USA). Data on carcass quality and incidences of condemned organs were analysed using Pearson's chi-square test and Fisher's exact test, respectively. Other data were analysed using the Mann–Whitney U test. Values of $P < 0.05$ were considered statistically significant.

1.3 Results

1.3.1 Environmental condition

Summer-finished pigs were reared under higher average whole-day temperatures and relative humidity than winter-finished pigs. Nocturnal temperatures were around 2°C lower than daytime temperatures for summer-finished pigs and around 4°C lower for winter-finished pigs. Average relative humidity varied within a narrow range for summer- and winter-finished pigs (Table 1-1).

1.3.2 Inspection of carcasses and internal organs

Data collected at slaughter were compared between summer-finished and winter-finished pigs (Table 1-2). Summer-finished pigs tended to have lower carcass quality ($P = 0.065$) and significantly greater back fat thickness. Incidences of condemned internal organs did not differ between summer-finished and winter-finished pigs.

1.3.3 Blood biochemistry and stress markers

In blood biochemistry (Table 1-3), summer-finished pigs showed significantly lower mean Na and Cl concentrations than those in winter-finished pigs and tended to have higher TP value (P = 0.052). Glb and A/G ratio also significantly differed between summer-finished and winter-finished pigs. On the other hand, there was no difference in blood stress markers in summer-finished and winter-finished pigs (Table 1-4).

The value of d-ROMs and BAP were examined as indicators of ROS production and antioxidant potential, respectively. The summer-finished pigs showed significantly higher d-ROMs than winter-finished pigs, although BAP and BAP/d-ROMs did not differ between the two seasonal cohorts (Table 1-5).

1.4 Discussion

In this study, I evaluated porcine heat stress in a subtropical region by comparing summer-finished and winter-finished pigs slaughtered at the end of a two-month finishing period. Also, I attempted to determine the relative characterisation of markers of heat stress and particularly of d-ROMs and BAP as indices of ROS derivates and antioxidant potential, respectively, in farm-reared pigs under seasonal conditions expected to produce chronic heat stress (summer) and thermoneutral temperatures (winter).

Based on the results of environmental temperature in the pig house and the large summer variance there from the thermoneutrality of the temperature range of 10 to 23.9°C (20) in which the winter-finished pigs were raised, it is considered that the summer-finished pigs experienced chronic heat stress. Previous studies have expanded the definition of acute and chronic heat stress in terms of exposure time and temperature for pigs (22, 39, 46). Reportedly, chronic heat stress has been induced under laboratory conditions in finishing pigs exposed to constant temperatures of 30°C or more for three weeks (21, 37). In this study, the summer-finished pigs experienced a slightly lower daytime temperature (around 29°C), with a further decrease in nocturnal hours, but for a more prolonged period (two months). Thus, it is considered that the conditions of the summer finishing period correspond to chronic heat stress.

The results of carcass quality assessment suggest a chronic heat stress effect. The greater back fat thickness in summer-finished pigs is considered to be a poorer result. More than 90% of the summer-finished pigs were measured at back fat thickness exceeding the upper limit of the criterion of the Japan Meat Grading Association (47). Although the difference in carcass quality was significant, this study results do not allow for definite conclusions on fat accumulation as a heat stress effect under field conditions. However, heat stress has been previously implicated in the upregulation of lipoprotein lipase (48) and fatty acid synthase activities (49), resulting in increased adipose tissue. Further research is needed to elucidate the mechanisms of heat stress-induced metabolic changes under field conditions, and to explain the subsequent alterations in carcass phenotype.

The differences between summer- and winter-finishing cohorts were considered to reflect adverse effects of seasonal heat stress, with Na and Cl levels suggestive of hypotonic dehydration; however, the blood biochemistry profiles of summer-finished pigs did not correspond to a state of disease. The data on blood chemistry and blood stress markers provide an interesting contrast to previous studies of chronic heat stress under laboratory conditions. Finishing pigs housed at 30°C for three weeks exhibited increased cortisol, lactate dehydrogenase (LDH), and creatinine kinase (CK) (22). The mean values of LDH and CK in this study were considerably higher in the summer-finished pigs, although the differences were not statistically significant. Data from this study implies that the adverse effect on muscle tissue was apparently not of sufficient magnitude to be detected in the measurements, including those of cortisol and other blood stress markers.

The presence of a chronic heat stress phenomenon in this study is further substantiated by the A/G ratio and SAA. The A/G ratio decreases with an increase in the globulin level, which implies chronic inflammation (50), and SAA is an acute-phase protein. The significantly lower A/G ratio in summer-finished pigs was not accompanied by any change in SAA. Taken together with the other biochemistry and stress marker results, this suggests that the seasonal heat stress was chronic, but not sufficiently severe to elevate stress marker levels. Furthermore, the A/G ratio might be a potential marker of chronic heat stress under field conditions.

Set against the results of blood biochemistry and stress markers, the novel findings on markers of blood oxidative stress in this study are of considerable interest. Short-term heat exposure reportedly increases oxidative stress marker levels (malondialdehyde, catalase activity and superoxide dismutase) in porcine skeletal muscles (39, 46), which could be consistent with the elevated plasma d-ROMs levels in this study. This elevation in d-ROMs was not accompanied by any increase in stress hormone levels, and it is considered that this change might be detectable before any changes in hormonal regulation of cortisol and other blood stress markers. Accordingly, d-ROMs might be more sensitive markers of heat stress than the blood stress markers previously suggested.

In summary of this Chapter, it was shown that pigs reared under subtropical field conditions are exposed to chronic heat stress, which negatively affects carcass quality. Futhermore, d-ROMs and the A/G ratio may be useful as sensitive markers of such heat stress under field conditions.

Table 1-1. Environmental temperature and relative humidity in the pig house

Group	Temperature (°C)			Relative humidity (%)		
	Whole day	Daytime (time)	Night (time)	Whole day	Daytime(time)	Night (time)
Summer[1]	28.3 ± 2.4	29.3 ± 2.6 (6:00–18:00)	27.1 ± 1.6 (19:00–5:00)	80.7 ± 6.4	80.5 ± 6.8 (6:00–18:00)	81.0 ± 6.0 (19:00–5:00)
Winter[2]	20.2 ± 4.9	22.2 ± 4.5 (7:00–17:00)	18.5 ± 4.6 (18:00–6:00)	71.7 ± 4.6	71.4 ± 4.7 (7:00–17:00)	71.9 ± 4.5 (18:00–6:00)

[1] Summer-finished pigs. The data for this group was recorded from July to September

[2] Winter-finished pigs. The data for this group was recorded from October to December

Table 1-2. Results of inspection of carcasses and incidences of internal organ condemnation

Group	Carcass result score*			Internal organs condemnation# (Incidence, %)		
	Result (Score$)	Back fat thickness (cm)	Weight (kg)	Heart	Liver	Stomach and intestines
Summer[1]	1.1 ± 0.3	4.1 ± 0.8	78.7 ± 3.3	0	50.0	41.7
Winter[2]	1.8 ± 0.9	2.5 ± 0.8	80.6 ± 4.3	0	50.0	33.3
P value	0.065	0.000	0.340		1.00	1.00

[1] Summer-finished pigs slaughtered from August to September

[2] Winter-finished pigs slaughtered from November to December

$ 3: high grade, 2: middle grade, 1: low grade

Data were analysed using Pearson's chi-square test (*) and Fisher's exact test (#)

Table 1-3. Common blood biochemistry analyte concentrations

Analyte	Unit	Summer[1]	Winter[2]	P value
LDH	U/L	1143.6 ± 829.8	737.9 ± 207.4	0.119
CK	U/L	11149.8 ± 23757.1	3778.4 ± 4034.7	0.453
TP	g/dL	8.2 ± 0.5	7.9 ± 0.6	0.052
Alb	g/dL	4.5 ± 0.4	4.8 ± 0.3	0.163
Glb	g/dL	3.7 ± 0.6	3.1 ± 0.5	0.015*
A/G	ratio	1.3 ± 0.3	1.6 ± 0.2	0.019*
Na	mEq/L	148.5 ± 4.0	151.8 ± 4.3	0.032*
Cl	mEq/L	98.6 ± 2.3	101.1 ± 3.4	0.048*

[1] Summer-finished pigs slaughtered from August to September

[2] Winter-finished pigs slaughtered from November to December

LDH: Lactate dehydrogenase; CK: Creatinine kinase; TP: Total protein; Alb: Albumin; Glb: Globulin; A/G: Albumin/globulin ratio

* $P < 0.05$: significantly different between summer and winter

Table 1-4. Blood stress marker concentrations

Marker	Unit	Summer[1]	Winter[2]	P value
Cortisol	µg/dL	4.6 ± 2.3	4.5 ± 2.4	0.931
Adrenalin	ng/mL	42.9 ± 15.7	46.2 ± 28.7	0.729
Noradrenaline	ng/mL	49.3 ± 19.9	41.2 ± 26.5	0.299
Dopamine	ng/mL	not detected	not detected	
Serum amyloid A	mg/L	6.0 ± 4.7	6.0 ± 5.1	0.773

[1] Summer-finished pigs slaughtered from August to September

[2] Winter-finished pigs slaughtered from November to December

Table 1-5. Blood oxidative stress

Parameter	Unit	Summer[1]	Winter[2]	P value
d-ROMs	U. CARR	1106.2 ± 136.6	970.8 ± 83.0	0.017*
BAP	μmol/L	4123.0 ± 771.7	4000.2 ± 306.4	0.356
BAP/d-ROMs		3.8 ± 0.9	4.1 ± 0.4	0.488

[1] Summer-finished pigs slaughtered from August to September

[2] Winter-finished pigs slaughtered from November to December

d-ROMs: derivatives of reactive oxygen metabolite-derived compounds (1 U. CARR = 0.08 mg H_2O_2/dL)

BAP: biological antioxidant potential

*P < 0.05: significantly different between summer and winter

CHAPTER2

Acupuncture treatment improves transport stress in microminipigs through the acupoint in ears

This work has been published as:

Ijiri M, Akioka K, Kitano T, Miura H, Ono HK, Terashima R, Fujimoto Y, Matsuo T, Yamato O, Kawaguchi H (2023). Acupuncture Treatment Improves Transport Stress in Microminipigs Through the Acupoint in Ears *In Vivo,* **37**, 2100-2104.

2.1 Introduction

It is well known that pigs are exposed to stress during transportation. An increase in plasma cortisol concentration has been found in connection with transport (51). The active response to stress is mainly associated with adrenal medulla and sympathetic nervous system (catecholamine) activation, whereas the passive response is hypothesized to be related to stimulation of the pituitary–adrenocortical system (37). Moreover, transport stress may be more or less severe depending on several factors, including crowding, temperature, and duration. However, measures against transport stress in pigs are insufficient and require further investigation.

Oriental medicines, including acupuncture, generally have fewer side effects than Western medicines. Therefore, acupuncture is useful for avoiding drug residue and side effect issues in laboratory animals and livestock. It has been reported that transdermal needles were applied to acupoints on both ears and successfully suppressed symptoms such as drooling and vomiting seen during transport (52).

In this study, I applied the canine transport stress-reducing acupuncture treatment on experimental microminipigs and investigated the effect of acupuncture treatment on transport stress to further animal welfare.

2.2 Materials and Methods

2.2.1 Animals

All microminipigs were obtained from one breeder (Fuji Micra Inc., Shizuoka, Japan). This research was performed in accordance with the Institutional Guidelines for Animal Experiments and in compliance with the Japanese Act on Welfare and Management of Animals (Act No. 105 and Notification No. 6).

2.2.2 Experiment 1 (Blood stress marker examination)

Six mature male microminipigs were used in this study. The animals were divided into two groups: control (n=3) and treatment (acupuncture treatment, n=3). In the treatment group, short, ultra-thin circular transdermal needles (Pyonex 1.5 mm, SEIRIN, Shizuoka, Japan, diameter × length = 0.2 × 1.5 mm) were applied to locations corresponding to the acupoints on the apical area of both ears, which are identified as Erjian Points one to three (“Jisen” in Japanese), approximately 30–60 min before transportation. All animals were transported from the production farm to the research facility in individual cages using transport vehicles and planes for approximately 6 h.

Peripheral blood samples were collected from the cranial vena cava 2 days before and immediately after transportation. The following stress markers were assessed in the blood: concentrations of cortisol using electrochemiluminescence immunoassay and catecholamines (including adrenaline and noradrenaline) using high-performance liquid chromatography. In the biochemical examination, the concentrations of serum amyloid A (SAA) and glucose were measured using a simple blood glucose meter (Nipro Stat Strip XP2, Nipro, Osaka, Japan) and non-esterified fatty acids (NEFA) were measured using NEFA C-Test Wako (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Furthermore, blood oxidative stress, derivatives of reactive oxygen metabolites (d-ROMs) concentrations, biological antioxidant potential (BAP), and the BAP/d-ROMs ratio were measured. The SAA concentrations and blood oxidative stress were measured as described below.

2.2.3 Measurement of SAA concentration

SAA concentration was measured using a Pentra C200 automated biochemical analyzer (HORIBA ABX SAS, Montpellier, France) with an SAA reagent specialized for animal serum or plasma (VET-SAA "Eiken" Reagent; Eiken Chemical Co., Ltd., Tokyo, Japan). Furthermore, SAA concentration was calculated based on a standard curve generated using a VET-SAA Calibrator Set (Eiken Chemical Co., Ltd.).

2.2.4 Measurement of blood oxidative stress

Serum d-ROMs and BAP levels represent reactive oxygen metabolites and antioxidant capacity, respectively. Serum levels of these two markers were measured using a FREE Carrio Duo Redox Analyzer system (Wismerll, Tokyo, Japan), which included a photometer and thermostatically regulated mini-centrifuge. Both d-ROMs and BAP tests were conducted according to manufacturer instructions.

2.2.5 Experiment 2 (Follow-up study: Incidence of diarrhea after transportation)

The incidence of diarrhea in microminipigs after transportation was investigated. Twenty animals were divided into four groups. The control (no treatment, n=2) and treatment groups (n=6) were subjected to a short duration of transportation (approximately 1 h using transport vehicle), and the other control (no treatment, n=6) and treatment groups (n=6) were subjected to a longer duration of transportation (approximately 6 h using transport vehicle and plane). Animals in both treatment groups were treated with acupuncture as in experiment 1. In addition to these four groups, 161 animals treated with acupuncture, as in experiment 1, were observed for diarrhea incidence after prolonged transportation (approximately 17 h using transport vehicle).

2.2.6 Statistical analysis

Blood examination data are expressed as mean ± standard deviation. Statistical analysis of the differences before and after treatment was performed using a paired *t*-test and that of the incidence was performed using Fisher's exact test. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (53). Values of $p<0.05$ were considered statistically significant.

2.3 Results

2.3.1 Experiment 1 (Blood stress marker examination)

Data from blood examinations before and after transportation are shown in Table 2-1. Regarding blood stress markers, the blood cortisol concentrations in the control and treatment groups after transportation showed higher values and tendencies compared with those before transportation. Blood adrenaline and noradrenaline concentrations did not increase compared with those before transportation.

As for biochemical examination, the SAA values in the control group after transportation increased approximately two-fold (rate of increase: 2.00 ± 0.71-fold) compared with those before transportation; concentrations in the treatment group did not increase (rate of increase: 1.04 ± 0.21-fold). Blood glucose concentrations were significantly higher in both groups after transportation than those before. Blood NEFA concentrations were higher after transportation than those before in all animals, and the mean value increased approximately three-fold after transportation.

Regarding oxidative stress, the blood concentrations of d-ROMs and BAP in both groups showed no significant changes before and after transportation. However, the d-ROMs values in the control group after transportation increased about 1.3-fold (rate of increase: 1.32 ± 0.42-fold) compared with those before transport; values in the treatment group did not increase (rate of increase: 1.02 ± 0.04-fold). The BAP/d-ROMs ratio in the control group after transportation was lower than that before transportation, whereas that in the treatment group showed no significant change.

2.3.2 Experiment 2 (Follow-up study: Incidence of diarrhea after transplantation)

As shown in Table 2-2, diarrhea incidence was 25% in the control group (total short and long transportation), whereas diarrhea was not observed during both short and long transportation in the treatment group.

2.4 Discussion

In this study, acupuncture was performed at the acupoints “Jisen”, which are one or three points on the outer blood vessel of the auricle apex and are anatomically located in the region innervated by the trigeminal nerve (52). Treatment stimulation also affects the center of the hypothalamus and vagus nerve and is thought to be affected by somatic autonomic reflexes, such as suppression and enhancement of gastric acid secretion.

Data on cortisol, a typical stress marker in pigs, suggest that acupuncture treatment has a stress-relieving effect. Results of previous studies assessing cortisol effectiveness in pigs as a parameter for stress quantification during transportation show controversial findings. For example, Piñeiro *et al*. reported that cortisol concentrations were not modified after 24 and 48 h of transport, indicating that cortisol levels may have returned to baseline during the long transportation (17). In the 6-h transportation conducted in this study, all individuals in both groups had elevated blood cortisol concentrations after transportation. Furthermore, a statistical comparison of the pre- and post-transportation values for each group showed significant differences only for the control group. However, this is insufficient to confirm the inhibitory effects of acupuncture on elevated blood cortisol levels; further research is required to confirm these effects.

Increased catecholamine levels due to elevated sympathetic nervous activity are known to cause a variety of physiological and emotional stress responses (13, 54). In addition, several studies have indicated that acupuncture modulates the activity of the locus coeruleus, which is the primary source of central noradrenergic neurons (55). On these grounds, I hypothesized that acupuncture treatment during transportation may relieve the extensive stress responses caused by the catecholaminergic system and contribute to animal well-being. However, no statistically significant differences in the peripheral catecholamine levels were observed before and after transportation in this study, and the effects of acupuncture could not be confirmed.

The transport stress phenomenon in this study was further investigated by analyzing blood SAA, glucose, and NEFA concentrations. SAA is an acute-phase protein (APP) identified as a positive acute-phase reactant (37). Glucose and its alternative fuels, NEFA, beta-hydroxybutyrate, and glycerol, can be used as indicators of metabolic stress induced by food and water restriction (13). Glucose is also affected by the sympathetic nervous system, and the hypothalamic-pituitary-adrenal (HPA) axis and could be an indicator of these responses (13). In this study, transportation stress induced an increase in blood SAA, glucose, and NEFA values, and acupuncture treatment alone suppressed the increase in blood SAA values. The increase in blood glucose and NEFA values was also considered to reflect transportation stress because of the increase in cortisol *via* HPA axis activation, whereas acupuncture treatment did not affect these changes. It has been reported that APPs, including SAA, increase after prolonged transportation of pigs for 12 h or longer (55). Longer transportation times and continuous post-transportation sampling may show an increase in SAA due to transportation stress and the effect of acupuncture treatment, warranting further study in the future.

The novel findings regarding blood oxidative stress markers in this study are of considerable interest. The d-ROMs are among the blood markers used to evaluate oxidative stress, and elevated levels have been observed in pigs reared under chronic heat stress as shown in Chapter 1. However, it is not necessarily accompanied by an increase in common stress markers, such as cortisol, and the causal relationship has not been clarified. Furthermore, the BAP/d-ROMs ratio is important because antioxidant reactions occur *in vivo* to resist oxidative stress. In this study, transportation stress induced a decrease in the BAP/d-ROMs ratio, while acupuncture treatment reversed this change. The decrease in the BAP/d-ROMs ratio owing to transportation stress was caused by an increase in the d-ROMs and remaining BAP values after transportation.

Therefore, it was thought that acupuncture treatment reduced transportation stress and, as a result, improved blood d-ROMs levels and the BAP/d-ROMs ratio.

Transportation stress is associated with increased gastrointestinal permeability, bacterial translocation, toxin production, and tissue damage as well as low immunity (56, 57). In experiment 2, diarrhea occurred in microminipigs without acupuncture treatment after transportation, considered to be because of transport stress. Pig transportation induces oxidative damage to the intestines, resulting in the destruction of intestinal integrity (56). Because acupuncture treatment suppressed diarrhea occurrence in microminipigs, it has been suggested that acupuncture suppresses diarrhea in other pig breeds. Taken together with the results of Experiment 1, the effects of transportation and acupuncture on blood SAA levels and oxidative stress may be related to intestinal oxidative stress. Although the effect of the autonomic nervous system on transport stress was not determined from the present data, it is well known that intestinal function and the autonomic nervous system are closely interrelated. Further research is required to clarify the mechanism of diarrhea induced by transport stress and validate the effectiveness of acupuncture treatment.

In summary of this Chapter, acupuncture treatment suppresses HPA axis activation, thereby reducing transport stress; however, it did not suppress central catecholaminergic or sympathetic activity in microminipigs.

Table 2-1. Blood examination

Parameter	Group	Before transportation	After transportation	*P* value*
[Stress Markers]				
Cortisol (ng/ml)	Control	2.1 ± 0.4	8.9 ± 2.5	<0.05
	Treatment	2.5 ± 0.8	12.8 ± 5.0	0.065
Adrenaline (ng/ml)	Control	91.3 ± 118.7	61.3 ± 42.0	0.609
	Treatment	113.0 ± 58.0	56.7 ± 21.5	0.118
Noradrenaline (ng/ml)	Control	186.3 ± 178.9	152.7 ± 90.0	0.588
	Treatment	233.3 ± 93.9	167.7 ± 38.4	0.281
[Biochemical Examination]				
Serum amyloid A (mg/l)	Control	0.94 ± 0.33	1.76 ± 0.63	0.155
	Treatment	0.94 ± 0.06	0.96 ± 0.15	0.830
Glucose (mg/dl)	Control	66.3 ± 7.6	92.7 ± 4.7	<0.05
	Treatment	69.7 ± 7.8	94.3 ± 2.5	<0.05
NEFA (mEq/l)	Control	0.20 ± 0.06	0.55 ± 0.23	0.156
	Treatment	0.19 ± 0.05	0.63 ± 0.27	0.126
[Oxidative Stress]				
d-ROMs (U.CARR)	Control	985.7 ± 273.9	1322.7 ± 422.2	0.098
	Treatment	842.3 ± 84.1	857.0 ± 97.2	0.523
BAP (μmol/l)	Control	3117.3 ± 309.1	3304.7 ± 616.7	0.435
	Treatment	3165.0 ± 141.8	3021.0 ± 138.8	0.408
BAP/d-ROMs ratio	Control	3.4 ± 1.1	2.7 ± 1.0	<0.05
	Treatment	3.8 ± 0.5	3.5 ± 0.3	0.321

*: Paired-samples t-test

Table 2-2. Incidence of diarrhea after transportation

	Control		Treatment	
	n	Diarrhea	n	Diarrhea
Short transportation	2	1 (50.0%)	6	0
Long transportation 1	6	1 (16.7%)	6	0
Long transportation 2	—		161	0
Total	8	2 (25.0%)	173	0**

Short transporation: within 1 hour, long transportation 1 and 2: 6 and 17 hours, respectively. **$p<0.01$: significantly different from control group.

GENERAL DISCUSSION

It is known that a variety of stressors are existing in the industrial pig farming environment and can induce a variety of biological responses, including behavioral, neurological, or immunological. In recent decades, there has been growing concern regarding stress among pigs because of the undesirable consequences that stress imposes on the normal physiology of pigs, their welfare, and general productive performance (13). For example, heat stress causes poor semen production and quality and quantity in boars and compromises normal embryo development in sows, resulting in a reduced farrowing rate, which is called seasonal infertility (17). Weaning and transportation are also major stresses for pigs, which leads to malnutrition, susceptibility to infection, deterioration of meat quality, and other spillover effects, causing a decline in productivity (13).

The expression of stress results primarily from two responses below. One is from activation of the sympathetic nervous system (SNS), in which catecholamines (epinephrine and norepinephrine) are secreted by the chromaffin cells of the adrenal medulla. An increase in peripheral catecholamines causes not only physiological stress responses but also deterioration of meat quality in domestic swine (13, 58). Additionally, elevated central noradrenergic activity causes psychological and emotional stress responses, such as anxiety, fear, and exaggerated aggressiveness (54). Furthermore, catecholamines increase the number of leukocytes, mainly neutrophils and lymphocytes, under acute stress. The another is hypothalamic-pituitary-adrenal (HPA) axis response, which is caused by a hormone-mediated hierarchical. That is, stressful stimulus causes the secretion of corticotropin-releasing hormone from the hypothalamus, followed by adrenocorticotropic hormone from the anterior pituitary gland, and finally glucocorticoids from the adrenal cortex. Cortisol is the major glucocorticoid in pigs and increases the catabolism of tissue to produce glucose, but also affects the reproductive and immune systems (13). Glucocorticoids inhibit the secretion of luteinizing hormone from the pituitary and various reproductive hormones from each gonad (59, 60). In the immune system, glucocorticoids suppress the production of cytokines and immunoglobulins (61). Additionally, prolonged exposure to stress and a predominance of cortisol results in a decrease in lymphocytes in the blood. Because of these mechanisms, there is a tendency to synthetically evaluate a variety of biomarkers representing different body systems such as SNS, HPA axis, reproductive and immune systems. In this study, catecholamines were used as biomarkers reflecting SNS. Cortisol is a widely used biomarker representative of the HPA axis, which was also measured in this study. Glucose is also affected by the SNS, and the HPA axis and could be an indicator of these responses (13).

Furthermore, prolonged stress can also affect metabolism and the immune system, and some studies have attempted to assess stress using various blood biochemical and immune system indices (13). Glucose and its alternative fuels, non-esterified fatty acids (NEFA), beta-hydroxybutyrate, and glycerol, can be used as indicators of metabolic stress induced by food and water restriction (13). Acute phase proteins (APPs), such as serum amyloid A (SAA), haptoglobin, and C-reactive protein, are recognized markers of inflammation and have also been proposed as indicators of stress in cattle and pigs (17, 38). Especially when stress is prolonged, oxidative stress is a consequence of any stress (62). Oxidative stress is defined as the balance between reactive oxygen species and the antioxidant system in the body, and it has been suggested that elevated oxidative stress increases oxidative damage, may be involved in various human disorders and pathogeneses (41, 63, 64). While various oxidative stress markers have been proposed as indicators for diagnosis and prevention of pathological condition, derivatives of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) were used as indicators in this study. These markers have been reported linking to various disease in human medicine (41, 63, 64), and obesity and metabolic syndrome are associated with low blood BAP/d-ROMs ratio (65).

In Chapter 1, I examined the affects of heat stress, which is considered to be the most concerning issue in Kagoshima prefecture. Kagoshima is located in the subtropical climate zone, and severe heat stress is expected to affect pigs during the summer months. Actually, the summer (June to September) temperatures in the pig house where this examination was conducted remained above the thermoneutral zone throughout the day. This indicates that the summer-finished pigs reared under subtropical field conditions in this study showed evidence of chronic heat stress, reflected in lower carcass quality than that of winter-finished pigs. In addition, increased backfat thickness has been confirmed resulting in a decrease in carcass value, which is considered to be an industrial issue. Heat stress alters metabolism, structure and antioxidant mechanisms in skeletal muscle (66). Heat exposure of sows during pregnancy affects fetal development and post-growth carcass quality (26, 67). In growing pigs, it has been reported that acute heat stress disrupts muscle redox balance and inflammatory signaling (39, 68). Heat stress has also been previously implicated in the upregulation of lipoprotein lipase (48) and fatty acid synthase activities (49), resulting in increased adipose tissue. Although the mechanism of backfat accumulation is not clear from the present study, these demonstrate the potential adverse effects of high temperature levels on the swine industry, and underlines the need for sensitive markers to monitor heat stress. The heat conditions described in Chapter 1 were probably chronic since the temperature conditions were milder and of longer duration than in other studies conducted in the laboratory. It is probably for this reason that many indicators, such as catecholamines and cortisol, did not show significant changes. However, d-ROMs value was elevated in the summer-finished pigs, suggesting the occurrence of oxidative stress. Since healthy pigs are expected to adapt to stress, and their owners generally try to provide appropriate environment, more sensitive markers are needed to measure stress in pigs in the rearing environment. Such markers would help in the development of environments and handling practices that are more consistent with animal welfare through stress assessments.

The effects of transport stress, considered one of the major stressors for pigs, were examined in Chapter 2. Additionally, the effectiveness of acupuncture on pigs as a method of reducing stress was evaluated. In contrast to that observed in the chronic heat environment (Chapter 1), increased blood cortisol and glucose concentrations were observed after transport, and SAA values increased approximately twofold after transport. On the other hand, a decrease in the BAP/d-ROMs ratio due to an increase in d-ROMs level was shown for oxidative stress. These effects were reduced in the acupuncture treatment group, suggesting that the effects of stress during transport were suppressed by acupuncture. An increase in peripheral catecholamines by elevated sympathetic nervous activity causes not only physiological stress responses but also deterioration of meat quality in domestic swine (13, 58). Additionally, elevated central noradrenergic activity causes psychological and emotional stress responses, such as anxiety, fear, and exaggerated aggressiveness (54). Because peripheral noradrenaline levels are known to correlate with central noradrenaline levels (69, 70), acupuncture treatment may also suppress transportation-induced increases in central noradrenaline levels. However, in the present study, there were no changes in peripheral catecholamine levels before or after transport. Furthermore, there were no differences with or without acupuncture treatment, and the involvement of the autonomic nervous system could not be proven. Treatment of the apex of the ear is generally effective against common cold, moderate heat, poisonings, febrile disease, indigestion, and shock. The acupoints used in present study are anatomically located in the region innervated by the trigeminal nerve (52), and it is thought that treatment stimulation also affects the center of the hypothalamus and vagus nerve and is influenced by somatic autonomic reflexes, such as suppression and enhancement of gastric acid secretion. Although the effect of the autonomic nervous system on transport stress was not determined from the present data, it is well known that intestinal function and the autonomic nervous system are closely interrelated. Further research is required to clarify the mechanism of diarrhea induced by transport stress and validate the effectiveness of acupuncture treatment.

In both studies, the pigs showed reactions that appeared to be caused by stress: degradation of carcass value in heat and diarrhea in transport. In transport, an increase in cortisol was detected after transportation, but other well-known biomarkers did not show obvious change. On the other hand, d-ROMs value, an oxidative stress marker, was suggested to be increased by exposure to stress in both tests. As shown in this study, the possibility of detecting potential stress suggests that this method may become a new tool for stress assessment. It has been proposed that d-ROMs is a marker that comprehensively assess oxidative stress in the body, and when combined with BAP, it can be used to assess the balance between oxidative and antioxidant stress (41, 65, 71, 72). In addition, it is considered to be suitable for veterinary applications because the target substance is relatively stable, and the measurement method is very simple (71, 72). However, there have been evaluated in a limited number of studies in domestic animals (42-45), and more detailed studies are needed.

With its involvement in the stress response, oxytocin is becoming increasingly important in stress research in humans and animals (73). Oxytocin is a peptide hormone primarily synthesized in magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus. The synthesized oxytocin is transported to the posterior pituitary gland, where it is released and involved in parturition and lactation, physiological effects of which have long been investigated (73). In recent decades, as the distribution of oxytocin receptors in the central nervous system has become clarified, it has become obvious that the effects of oxytocin are diverse, including maternal behavior, social recognition, feeding behavior, and stress regulation. Regarding the stress-regulating function of oxytocin, it has been reported that it is involved in the regulation of stress responses *via* HPA axis (73) and is thought to work as an anxiolytic as it decreases release of stress hormones (74). Because oxytocin appears to be involved in behavioral physiology and stress regulation, it has gained high interest as a biomarker for welfare assessment in farm animals. Oxytocin has also been reported to be analyzed in samples that can be collected noninvasively, such as saliva and hair, which is considered a great advantage (75, 76). In the veterinary field, injectable preparations of oxytocin are used mainly to stimulate lactation and induce farrowing. However, these preparations are not administrated to livestock for improving animal welfare, and it seems would not be an impractical strategy. Similarly, acupuncture also appears to be very difficult to apply in the swine industry. Although acupuncture was found to be effective to a certain extent in this study, it is labor intensive to implement in actual pig farming. Materials that can be administered orally would be more practical for application in swine production.

Oxytocin is a peptide hormone and is not absorbed when administered orally. However, 1,5-anhydro-d-fructose (1,5-AF) has been reported as a substance that may increase oxytocin secretion endogenously (77). The 1,5-AF is a saccharide that has been isolated from several fungi (78) and an edible seaweed (79) as an intermediate of the microthecin biosynthesis pathway. This saccharide is considered to be widely distributed among organisms, including animals (80) Furthermore, 1,5-AF is produced as a food material in Japan *via* the enzymatic degradation of starch. The 1,5-AF has antioxidant (81) and antibacterial properties (82) and health benefits (83), thus it has utility as a food preservative and healthy food material. One very unique functionality has been reported that mice orally administered 1,5-AF show feeding suppression and that activation of oxytocin neurons may be involved in this satiety effect (77). Although these various functionalities have been reported, research is still in progress to determine whether these effects can be seen in humans and livestock as well. The 1,5-AF has been reported to be metabolized to 1,5-anhydro-d-glucitol (1,5-AG) in the body and excreted though the urine in mice (84). The 1,5-AG is present in the blood of humans and rats (85). The blood 1,5-AG concentration is clinically measured because it is correlated with blood glucose levels and is used in blood glucose control. The 1,5-AG also has beneficial properties for human health such as anti-inflammatory (86), blood glucose regulation in diabetes (87), and an inhibition of COVID-19 infection (88). Recently, it was reported that in humans and pigs, as in mice, it is metabolized to 1,5-AG in the body when administered orally (89). These findings indicate the potential of 1,5-AF as a stress-reducing material, while further detailed investigation of its effects and metabolism in pigs is needed.

Stress conditions are generally managed by improving hardware such as facilities and changing husbandry methods, but there are some limitations to this approach alone. Stress reduction methods such as acupuncture and the administration of functional substances such as 1,5-AF are thought to provide a new perspective on improving animal welfare. Clarifying the physiological effects of 1,5-AF and its mechanism of action will contribute to the reduction of stress in farming animals. In addition to the existence of various stressors in the environment for farming pigs, the treatment of animals for evaluation their stress such as holding or blood sampling may also be a cause of stress, and further research, including methods for stress evaluation, is desirable for animal welfare.

SUMMARY AND CONCLUSION

As global demand for food increases, intensive livestock production is becoming more common, which is known to cause various stress-related health problems in animals. This not only directly affects livestock productivity, but in recent years, with the growing interest in animal welfare, it is desirable to develop a method of feeding livestock with less stress on them. On the other hand, although various biomarkers have been proposed to evaluate stress in livestock, there is no gold standard procedure, indicating the difficulty of stress assessment in livestock. In this dissertation, I focused on the effects of stress during swine feeding and management.

In Chapter 1, the effects of seasonal heat stress in Kagoshima Berkshire pigs reared in the subtropical region were investigated by comparing the biomarker changes in the summer and winter seasons. Pigs were allocated to summer- and winter-finishing cohorts, 12 each. The evaluations included assessment of carcass traits and internal organs' normality carried out at the time of slaughter, and measurement of biomarkers in whole blood: derivatives of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) as markers of oxidative stress, and serum amyloid A (SAA) and albumin/globulin (A/G) ratio as markers of acute and chronic inflammation, respectively. During the summer, the feeding environment in Kagoshima exceeded the thermal neutral zone for pigs, suggesting that the pigs were exposed to chronic heat stress. In winter, on the other hand, the pigs were within the thermal neutral zone throughout the rearing period. In addition, the summer-finished pigs reared under subtropical field conditions showed lower carcass quality than the winter-finished pigs, indicating a potential adverse effect of summer temperatures on the swine industry. Marginal changes were observed in d-ROMs and the A/G ratio between the summer- and winter-finishing cohorts. The results demonstrated that d-ROMs and the A/G ratio could be used as sensitive markers for heat stress under field conditions.

In Chapter 2, the effects of transport stress were examined using experimental microminipigs, and the stress-reducing effects of acupuncture via ear acupuncture points were evaluated. The effects of transport and acupuncture treatment on pigs were examined using blood stress marker. Six animals were equally divided into two groups (Control and Treatment). In the treatment group, before transportation (6 h; vehicle and plane), short, ultrathin circular transdermal needles were applied to locations corresponding to the acupoints on the apical area of both ears. Peripheral blood samples were collected from the cranial vena cava 2 days before and immediately after transportation. Blood stress markers, biochemistry indicators, and oxidative stress levels were examined. Transport stress induced an increase in blood cortisol, SAA, glucose, non-esterified fatty acid, and d-ROMs and decreased the BAP/d-ROMs ratio yet did not affect BAP. Acupuncture suppressed the increases in SAA and d-ROMs values and the decrease in BAP/d-ROMs ratio. As a follow-up study, diarrhea incidences after transportation in the control and treatment groups were also investigated. The total diarrhea incidence was 25% in the control group, whereas diarrhea was not observed in the treatment group. These results suggest that acupuncture treatment suppresses hypothalamic–pituitary–adrenal function and, as a result, reduces transport stress without affecting the suppression of the central catecholaminergic system. Therefore, acupuncture treatment for transport stress was considered to have the potential to improve animal welfare. In summary, this dissertation verifies the effects of heat and transport stress on pigs and demonstrates the effectiveness of acupuncture as a method of reducing these effects. These should contribute to the development of livestock stress research and the establishment of strategies to manage stress in pigs.

Stress in the actual feeding environment did not necessarily show obvious changes in stress markers, but it was suggested to affect health status and meat quality. Furthermore, d-ROMs and BAP, indicators of oxidative stress, are sensitive markers and may be useful in assessing stress in livestock. The development of simpler and easier stress assessment methods is essential for livestock stress research and further study is desirable. In addition, the effects of acupuncture shown in this study provide a new perspective on stress management in livestock. Recently, functional saccharide named 1,5-anhydro-d-fructose (1,5-AF) was reported to increase endogenous oxytocin secretion in mice, suggesting that it may be effective in reducing stress in humans and animals. Since it has been suggested that 1,5-AF may be effective with oral administration, 1,5-AF is expected to be easily applied to livestock, including pigs. Stress reduction methods such as acupuncture and the administration of functional substances such as 1,5-AF are revolutionary in that they can contribute to stress reduction without requiring changes in equipment or husbandry methods.

ACKNOWLEDGEMENTS

I would like to express my profound regards and deepest sense of gratitude to my supervisor, Dr. Tomohide Matsuo, and my co-supervisors, Dr. Mitsuhiro Takagi, Dr. Akikazu Fujita, Dr. Naoki Miura, Dr. Yoshikazu Fujimoto, for kindly providing me the opportunity of higher study. I gratefully acknowledge their invaluable instructions and constructive criticism during the preparation of my doctoral dissertation.

It gives me great pleasure to express highly appreciations and heartfelt sense of gratitude to Dr. Hiroaki Kawaguchi, for his excellent and continuous supervision to my study, encouragement, and understanding.

I am grateful to all of my co-authors in my publications for their special suggestion to improve my manuscript: Dr. Osamu Yamato, Mr. Kenji Odo, Dr. Motohiko Sato, Dr. De-Xing Hou, Mr. Takashi Tanabe, Mr. Kohei Akioka, Dr. Taisuke Kitano, Dr. Hiroshi Miura, Dr. Hisaya Ono and Dr. Ryota Terashima.

Special thanks to Dr. Kazuhiro Yoshinaga, Ms. Maiko Kawaguchi, Ms. Shiho Suzuki, Mr. Naoki Nogami and Mr. Yoshiaki Shimoo for cooperation in carrying out the study. Special thanks are also expressed to Mr. Henry Smith for pertinent English

revision of the manuscript.

REFFERENCES

1. Frantz, L., Meijaard, E., Gongora, J., Haile, J., Groenen, M. A., Larson, G. (2016) The evolution of Suidae. *Annu Rev Anim Biosci.,* **4**, 61-85.
2. King, J. W. B., Roberts, R. C. (1960) Carcass length in the bacon pig; its association with vertebrae numbers and prediction from radiographs of the young pig. *Animal Production* **2**, 59-65.
3. Mikawa, S., Morozumi, T., Shimanuki, S., Hayashi, T., Uenishi, H., Domukai, M., Okumura, N., Awata, T. (2007) Fine mapping of a swine quantitative trait locus for number of vertebrae and analysis of an orphan nuclear receptor, germ cell nuclear factor (NR6A1). *Genome Res.* **17**, 586-593.
4. Mikawa, S., Hayashi, T., Nii, M., Shimanuki, S., Morozumi, T., Awata, T. (2005) Two quantitative trait loci on Sus scrofa chromosomes 1 and 7 affecting the number of vertebrae. *J Anim Sci.* **83**, 2247-2254.
5. Kawaguchi, H., Miyoshi, N., Miura, N., Fujiki, M., Horiuchi, M., Izumi, Y., Miyajima, H., Nagata, R., Misumi, K., Takeuchi, T., Tanimoto, A., Yoshida, H. (2011) Microminipig, a non-rodent experimental animal optimized for life science research: novel atherosclerosis model induced by high fat and cholesterol diet. *J Pharmacol Sci.* **115**, 115-121.

6. Kawaguchi, H., Yamada, T., Miura, N., Ayaori, M., Uto-Kondo, H., Ikegawa, M., Noguchi, M., Wang, K. Y., Izumi, H., Tanimoto, A. (2014) Rapid development of atherosclerosis in the world's smallest Microminipig fed a high-fat/high-cholesterol diet. *J Atheroscler Thromb.* **21**, 186-203.

7. Kawaguchi, H., Yamada, T., Miura, N., Noguchi, M., Izumi, H., Miyoshi, N., Tanimoto, A. (2013) Sex differences of serum lipid profile in novel microminipigs. *In Vivo.* **27**, 617-621.

8. Kawaguchi, H., Yamada, T., Miura, N., Takahashi, Y., Yoshikawa, T., Izumi, H., Kawarasaki, T., Miyoshi, N., Tanimoto, A. (2012) Reference values of hematological and biochemical parameters for the world smallest microminipigs. *J Vet Med Sci.* **74**, 933-936.

9. Miura, N., Kawaguchi, H., Nagasato, T., Yamada, T., Ito, T., Izumi, H., Shameshima, H., Miyoshi, N., Tanimoto, A., Maruyama, I. (2013) Coagulation activity and white thrombus formation in the microminipig. *In Vivo.* **27**, 357-361.

10. Takeishi, K., Kawaguchi, H., Akioka, K., Noguchi, M., Arimura, E., Abe, M., Ushikai, M., Okita, S., Tanimoto, A., Horiuchi, M. (2018) Effects of dietary and lighting conditions on diurnal locomotor activity and body temperature in microminipigs. *In Vivo.* **32**, 55-62.

11. Hamada, T., Watanabe, Y., Iida, K., Sano, N., Amano, N. (2020) Microminipig: A suitable animal model to estimate oral absorption of sustained-release formulation in humans. *Int J Pharm.* **584**, 119457.

12. Stricker-Krongrad, A., Shoemake, C. R., Bouchard, G. F. (2016) The miniature swine as a model in experimental and translational medicine. *Toxicol Pathol.* **44**, 612-623.

13. Martínez-Miró, S., Tecles, F., Ramón, M., Escribano, D., Hernández, F., Madrid, J., Orengo, J., Martínez-Subiela, S., Manteca, X., Cerón, J. J. (2016) Causes, consequences and biomarkers of stress in swine: an update. *BMC Vet Res.* **12**, 171.

14. Lallès, J. P., David, J. C. (2011) Fasting and refeeding modulate the expression of stress proteins along the gastrointestinal tract of weaned pigs. *J Anim Physiol Anim Nutr (Berl).* **95**, 478-488.

15. Ott, S., Soler, L., Moons, C. P., Kashiha, M. A., Bahr, C., Vandermeulen, J., Janssens, S., Gutiérrez, A. M., Escribano, D., Cerón, J. J., Berckmans, D., Tuyttens, F. A., Niewold, T. A. (2014) Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs. *Res Vet Sci.* **97**, 124-128.

16. Toscano, M. J., Lay, D. C. Jr., Craig, B. A., Pajor, E. A. (2007) Assessing the adaptation of swine to fifty-seven hours of feed deprivation in terms of behavioral and physiological responses. *J Anim Sci.* **85**, 441-451.

17. Piñeiro, M., Piñeiro, C., Carpintero, R., Morales, J., Campbell, F. M., Eckersall, P. D., Toussaint, M. J., Lampreave, F. (2007) Characterisation of the pig acute phase protein response to road transport. *Vet J.* **173**, 669-674.

18. Song, C., Jiang, J., Han, X., Yu, G., Pang, Y. (2014) Effect of immunological stress to neuroendocrine and gene expression in different swine breeds. *Mol Biol Rep.* **41**, 3569-3576.

19. Mayorga, E. J., Renaudeau, D., Ramirez, B. C., Ross, J. W., Baumgard, L. H. (2018) Heat stress adaptations in pigs. *Anim Front.* **9**, 54-61.

20. Myer, R., Bucklin, R. (2001) *Influence of hot-humid environment on growth performance and reproduction of swine (Document AN107).* Gainesville, FL: Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

21. Cui, Y., Gu, X. (2015) Proteomic changes of the porcine small intestine in response to chronic heat stress. *J Mol Endocrinol.* **55**, 277-293.

22. Hao, Y., Feng, Y., Yang, P., Feng, J., Lin, H., Gu, X. (2014) Nutritional and physiological responses of finishing pigs exposed to a permanent heat exposure during three weeks. *Arch Anim Nutr.* **68**, 296-308.

23. Serviento, A. M., Labussière, E., Castex, M., Renaudeau, D. (2020) Effect of heat stress and feeding management on growth performance and physiological responses of finishing pigs. *J Anim Sci.* **98**, skaa387.

24. Baumgard, L. H., Rhoads, R.P. Jr. (2013) Effects of heat stress on postabsorptive metabolism and energetics. *Annu Rev Anim Biosci.* **1**, 311-337.

25. Ross, J. W., Hale, B. J., Gabler, N. K., Rhoads, R. P., Keating, A. F., Baumgard, L. H. (2015) Physiological consequences of heat stress in pigs. *Animal Production Science* **55**, 1381-1390

26. Tuell, J. R., Nondorf, M. J., Maskal, J. M., Johnson, J.S., Kim, Y. H. B. (2021) Impacts of in utero heat stress on carcass and meat quality traits of market weight gilts. *Animals (Basel)*. **11**, 717.

27. Brown, S. N., Knowles, T. G., Wilkins, L. J., Chadd, S. A., Warriss, P. D. (2005) The response of pigs to being loaded or unloaded onto commercial animal transporters using three system. *Vet J.* **170**, 91-100.

28. Warriss, P. D. (1998) The welfare of slaughter pigs during transport. *Anim Welfare* **7**, 365-381.

29. Flores-Peinado, S., Mota-Rojas, D., Guerrero-Legarreta, I., Mora-Medina, P., Cruz-Monterrosa, R., Gómez-Prado, J., Guadalupe Hernández, M., Cruz-Playas, J., Martínez-Burnes, J. (2020) Physiological responses of pigs to preslaughter handling: infrared and thermal imaging applications. *Int J Vet Sci Med.* **8**, 71-84.

30. Oliván, M., González, J., Bassols, A., Díaz, F., Carreras, R., Mainau, E., Arroyo, L., Peña, R., Potes, Y., Coto-Montes, A., Hollung, K., Velarde, A. (2018) Effect of sex and RYR1 gene mutation on the muscle proteomic profile and main physiological biomarkers in pigs

at slaughter. *Meat Sci.* **141**, 81-90.

31. Čobanović, N., Čalović, S., Suvajdžić, B., Grković, N., Stanković, S. D., Radaković, M., Spariosu, K., Karabasil, N. (2024) Consequences of transport conditions on the welfare of slaughter pigs with different health status and RYR-1 genotype. *Animals (Basel).* **14**, 191.

32. Rey-Salgueiro, L., Martinez-Carballo, E., Fajardo, P., Chapela, M. J., Espiñeira, M., Simal-Gandara, J. (2018) Meat quality in relation to swine well-being after transport and during lairage at the slaughterhouse. *Meat Sci.* **142**, 38-43.

33. Guàrdia, M. D., Estany, J., Balasch, S., Oliver, M. A., Gispert, M., Diestre, A. (2004) Risk assessment of PSE condition due to pre-slaughter conditions and RYR1 gene in pigs. *Meat Sci.* **67**, 471-478.

34. Moberg, G. P., Mench, J. A. (2000) *The biology of animal stress: basic principles and implications for animal welfare.* USA: CABI Publishing.

35. Einarsson, S., Brandt, Y., Lundeheim, N., Madej, A. (2008) Stress and its influence on reproduction in pigs: a review. *Acta Vet Scand.* **50**, 48.

36. Starkie, R. L., Hargreaves, M., Rolland, J., Febbraio, M. A. (2005) Heat stress, cytokines, and the immune response to exercise. *Brain Behav Immun.* **19**, 404-412.

37. Cui, Y., Wang, C., Hao, Y., Gu, X., Wang, H. (2019) Chronic heat stress induces acute phase responses and serum metabolome changes in finishing pigs. *Animals (Basel).* **9**, 395.

38. Lomborg, S. R., Nielsen, L. R., Heegaard, P. M., Jacobsen, S. (2008) Acute phase proteins in cattle after exposure to complex stress. *Vet Res Commun.* **32**, 575-582.

39. Montilla, S. I., Johnson, T. P., Pearce, S. C., Gardan-Salmon, D., Gabler, N. K., Ross, J. W., Rhoads, R. P., Baumgard, L. H., Lonergan, S. M., Selsby, J. T. (2014) Heat stress causes oxidative stress but not inflammatory signaling in porcine skeletal muscle. *Temperature (Austin).* **1**, 42-50.

40. Akbarian, A., Michiels, J., Degroote, J., Majdeddin, M., Golian, A., De Smet, S. (2016) Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. *J Anim Sci Biotechnol.* **7**, 37.

41. Fukui, T., Yamauchi, K., Maruyama, M., Yasuda, T., Kohno, M., Abe, Y. (2011) Significance of measuring oxidative stress in lifestyle-related diseases from the viewpoint of correlation between d-ROMs and BAP in Japanese subjects. *Hypertens Res.* **34**, 1041-1045.

42. Otomaru, K., Oishi, S., Fujimura, Y., Iwamoto, Y., Nagai, K., Ijiri, M. (2020) Effects of vitamin C supplementation on the blood oxidative stress and antibody titre against *Histophilus Somni* vaccination in calves. *J Vet Res.* **65**, 73-78.

43. Sauerwein, H., Schmitz, S., Hiss, S. (2005) The acute phase protein haptoglobin and its relation to oxidative status in piglets undergoing weaning-induced stress. *Redox Rep.* **10**,

295-302.

44. Shono, S., Gin, A., Minowa, F., Okubo, K., Mochizuki, M. (2020) The oxidative stress markers of horses—the comparison with other animals and the influence of exercise and disease. *Animals (Basel).* **10**, 617.

45. Yun, W., Song, M., Lee, J., Oh, H., An, J., Kim, G., Lee, S., Lee, S., Kim, H. B., Cho, J. (2020) Arginine addition in a diet for weaning pigs can improve the growth performance under heat stress. *J Anim Sci Technol.* **62**, 460-467.

46. Volodina, O., Ganesan, S., Pearce, S. C., Gabler, N. K., Baumgard, L. H., Rhoads, R. P., Selsby, J. T. (2017) Short-term heat stress alters redox balance in porcine skeletal muscle. *Physiol Rep.* **5**, e13267.

47. Japan Meat Grading Association (1996) *Instruction manual of trading standard on carcass of cattle and swine, partial meat of cattle and swine (in Japanese).* Tokyo: Japanese Meat Grading Association.

48. Kouba, M., Hermier, D., Le Dividich, J. (2001) Influence of a high ambient temperature on lipid metabolism in the growing pig. *J Anim Sci.* **79**, 81-87.

49. Pearce, S. C., Gabler, N. K., Ross, J. W., Escobar, J., Patience, J. F., Rhoads, R. P., Baumgard, L. H. (2013) The effects of heat stress and plane of nutrition on metabolism in growing pigs. *J Anim Sci.* **91**, 2108-2118.

50. Maeda, S., Takeya, Y., Oguro, R., Akasaka, H., Ryuno, H., Kabayama, M., Yokoyama, S., Nagasawa, M., Fujimoto, T., Takeda, M., Onishi-Takeya, M., Itoh, N., Takami, Y., Yamamoto, K., Sugimoto, K., Inagaki, H., Ogawa, M., Nakagawa, T., Yasumoto, S., Masui, Y., Arai, Y., Ishizaki, T., Ikebe, K., Gondo, Y., Kamide, K., Rakugi, H. (2019) Serum albumin/globulin ratio is associated with cognitive function in community-dwelling older people: The Septuagenarians, Octogenarians, Nonagenarians Investigation with Centenarians study. *Geriatr Gerontol Int.* **19**, 967-971.

51. Dalin, A-M., Magnusson, U., Haggendal, J., Nyberg, L. (1993) The effect of transport stress on plasma levels of catecholamines, cortisol, corticosteroid-binding globulin, blood cell count, and lymphocyte proliferation in pigs. *Acta Vet Scand.* **34**, 59-68.

52. Kawaguchi, H., Sasatake, Y., Noguchi, M., Akioka, K., Miura, N., Takeishi, K., Horiuchi, M., Tanimoto, A. (2016) Study of a novel acupuncture treatment for canine transportation stress. *J Jpn Vet Med Assoc.* **69**, 143-146

53. Kanda Y. Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics. 2013 *Bone Marrow Transplant.* 248:452-458.

54. Yamamoto K, Shinba T, Yoshii M. Psychiatric symptoms of noradrenergic dysfunction: a pathophysiological view. 2014 *Psychiatry Clin Neurosci.* 68: 1-20.

55. Lee, G., Kim, W. (2017) The modulatory effect of acupuncture on the activity of locus

coeruleus neuronal cells: A review. *Evid Based Complement Alternat Med.* **2017**, 9785345.

56. He, Y., Sang, Z., Zhuo, Y., Wang, X., Guo, Z., He, L., Zeng, C., Dai, H. (2019) Transport stress induces pig jejunum tissue oxidative damage and results in autophagy/mitophagy activation. *J Anim Physiol Anim Nutr (Berl).* **103**, 1521-1529.
57. Zou, Y., Wei, H. K., Xiang, Q. H., Wang, J., Zhou, Y. F., Peng, J. (2016) Protective effect of quercetin on pig intestinal integrity after transport stress is associated with regulation oxidative status and inflammation. *J Vet Med Sci.* **78**, 1487-1494.
58. Foury, A., Devillers, N., Sanchez, M. P., Griffon, H., Le Roy, P., Mormède, P. (2005) Stress hormones, carcass composition and meat quality in Large White×Duroc pigs. *Meat Sci.* **69**, 703-707.
59. Chrousos, G. P., Torpy, D. J., Gold, P. W. (1998) Interactions between the hypothalamic-pituitary-adrenal axis and the female reproductive system: clinical implications. *Ann Intern Med.* **129**, 229-240.
60. Norman, R. L., Smith, C. J. (1992) Restraint inhibits luteinizing hormone and testosterone secretion in intact male rhesus macaques: effects of concurrent naloxone administration. *Neuroendocrinology.* **55**, 405-415.
61. Dhabhar, F. S. (2009) Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. *Neuroimmunomodulation.* **16**,

300-317.

62. Giergiel, M., Olejnik, M., Jabłoński, A., Posyniak, A. (2021) The markers of stress in swine oral fluid. *J Vet Res.* **65**, 487-495.

63. Hirata, Y., Yamamoto, E., Tokitsu, T., Fujisue, K., Kurokawa, H., Sugamura, K., Sakamoto, K., Tsujita, K., Tanaka, T., Kaikita, K., Hokimoto, S., Sugiyama, S., Ogawa, H. (2015) The pivotal role of a novel biomarker of reactive oxygen species in chronic kidney disease. *Medicine (Baltimore).* **94**, e1040.

64. Hirata, Y., Yamamoto, E., Tokitsu, T., Kusaka, H., Fujisue, K., Kurokawa, H., Sugamura. K., Maeda, H., Tsujita, K., Kaikita, K., Hokimoto, S., Sugiyama, S., Ogawa, H. (2015) Reactive oxygen metabolites are closely associated with the diagnosis and prognosis of coronary artery disease. *J Am Heart Assoc.* **4**, e001451.

65. Faienza, M. F., Francavilla, R., Goffredo, R., Ventura, A., Marzano, F., Panzarino, G., Marinelli, G., Cavallo, L., Di Bitonto, G. (2012) Oxidative stress in obesity and metabolic syndrome in children and adolescents. *Horm Res Paediatr* **78**, 158-164

66. Cruzen, S. M., Pearce, S. C., Baumgard, L. H., Gabler, N. K., Huff-Lonergan, E., Lonergan, S. M. (2015) Proteomic changes to the sarcoplasmic fraction of predominantly red or white muscle following acute heat stress. *J Proteomics.* **128**, 141-153.

67. Cruzen, S. M., Boddicker, R. L., Graves, K. L., Johnson, T. P., Arkfeld, E. K., Baumgard, L.

H., Ross, J. W., Safranski, T. J., Lucy, M.C., Lonergan, S. M. (2015) Carcass composition of market weight pigs subjected to heat stress in utero and during finishing. *J Anim Sci.* 93: 2587-2596.

68. Ganesan, S., Reynolds, C., Hollinger, K., Pearce, S. C., Gabler, N. K., Baumgard, L. H., Rhoads, R. P., Selsby, J. T. (2016) Twelve hours of heat stress induces inflammatory signaling in porcine skeletal muscle. *Am J Physiol Regul Integr Comp Physiol.* **310**, R1288-R1296.

69. Roy, A., Pickar, D., De Jong, J., Karoum, F., Linnoila, M. (1988) Norepinephrine and its metabolites in cerebrospinal fluid, plasma, and urine. Relationship to hypothalamic-pituitary-adrenal axis function in depression. *Arch Gen Psychiatry.* **45**, 849-857.

70. Ziegler, M. G., Lake, C. R., Wood, J. H., Brooks, B. R., Ebert, M. H. (1977) Relationship between norepinephrine in blood and cerebrospinal fluid in the presence of a blood-cerebrospinal fluid barrier for norepinephrine. *J Neurochem.* **28**, 677-679.

71. Pasquini, A., Luchetti, E., Marchetti, V., Cardini, G., Iorio, E. L. (2008) Analytical performances of d-ROMs test and BAP test in canine plasma. Definition of the normal range in healthy Labrador dogs. *Vet Res Commun.* **32**, 137-143.

72. Celi, P., Sullivan, M., Evans, D. (2010) The stability of the reactive oxygen metabolites

(d-ROMs) and biological antioxidant potential (BAP) tests on stored horse blood. *Vet J.* **183**, 217-218.

73. Lee, H. J., Macbeth, A. H., Pagani, J. H., Young, W. S. 3rd. (2009) Oxytocin: the great facilitator of life. *Prog Neurobiol.* **88**, 127-151.

74. Sikich, L., Kolevzon, A., King, B. H., McDougle, C. J., Sanders, K. B., Kim, S. J., Spanos, M., Chandrasekhar, T., Trelles, M. D. P., Rockhill, C. M., Palumbo, M. L., Witters Cundiff, A., Montgomery. A., Siper, P., Minjarez, M., Nowinski, L. A., Marler, S., Shuffrey, L. C., Alderman, C., Weissman, J., Zappone, B., Mullett, J. E., Crosson, H., Hong, N., Siecinski, S. K., Giamberardino, S. N., Luo, S., She, L., Bhapkar, M., Dean, R., Scheer, A., Johnson, J. L., Gregory, S. G., Veenstra-Vander Weele, J. (2021) Intranasal oxytocin in children and adolescents with autism spectrum disorder. *N Engl J Med.* **385**, 1462-1473.

75. López-Arjona, M., Tecles, F., Mateo, S. V., Contreras-Aguilar, M. D., Martínez-Miró, S., Cerón, J. J., Martínez-Subiela, S. (2021) A procedure for oxytocin measurement in hair of pig: Analytical validation and a pilot application. *Biology (Basel).* **10**, 527.

76. Valros, A., Lopez-Martinez, M. J., Munsterhjelm, C., Lopez-Arjona, M., Ceron, J. J. (2022) Novel saliva biomarkers for stress and infection in pigs: Changes in oxytocin and procalcitonin in pigs with tail-biting lesions. *Res Vet Sci.* **153**, 49-56.

77. Nakata, M., Yamaguchi, Y., Monnkawa, H., Takahashi, M., Zhang, B., Santoso, P., Yada, T.,

Maruyama, I. (2023) 1,5-Anhydro-D-fructose exhibits satiety effects via the activation of oxytocin neurons in the paraventricular nucleus. *Int J Mol Sci.* **24**, 8248.

78. Deffieux, G., Baute, R., Baute, M., Atfani, M., Carpy, A. (1987) 1,5-D-anhydrofructose, the precursor of the pyrone microthecin in morchella vulgaris. *Phytochemistry.* **26**, 1391-1393.

79. Broberg, A., Kenne, L., Pedersén, M. (1999) Analysis of 1,5-anhydro-D-fructose, microthecin, and 4-deoxy-glycero-hexo-2,3-diulose in algae using gas chromatography-mass spectrometry in selected ion monitoring mode. *Anal Biochem.* **268**, 35-42.

80. Kametani, S., Shiga, Y., Akanuma, H. (1996) Hepatic production of 1,5-anhydrofructose and 1,5-anhydroglucitol in rat by the third glycogenolytic pathway. *Eur J Biochem.* **242**, 832-838.

81. Fujisue, M., Yoshinaga, K., Muroya, K., Abe, J., Hizukuri, S. (1999) Preparation and antioxidative activity of 1,5-anhydrofructose. *J. Appl Glycosci.* **46**, 439-444.

82. Izumi S, Yoshinaga K, Abe J. (2013) Growth restraint action of 1,5-anhydro-D-fructose on gram-positive bacteria. *J. Appl Glycosci.* **60**, 87-93.

83. Fiskesund, R., Abeyama, K., Yoshinaga, K., Abe, J., Yuan, Y., Yu, S. (2010) 1,5-anhydro-D-fructose and its derivatives: biosynthesis, preparation and potential medical

applications. *Planta Med.* **76**, 1635-1641.

84. Yu, S., Mei, J., Ahrén, B. (2004) Basic toxicology and metabolism studies of 1,5-anhydro-D-fructose using bacteria, cultured mammalian cells, and rodents. *Food Chem Toxicol.* **42**, 1677-1686.

85. Dworacka, M., Winiarska, H., Szymanska, M., Kuczynski, S., Szczawinska, K., Wierusz-Wysocka, B. (2002) 1,5-anhydro-D-glucitol: a novel marker of glucose excursions. *Int J Clin Pract Suppl.* **(129)**, 40-44.

86. Meng, X., Tancharoen, S., Kawahara, K. I., Nawa, Y., Taniguchi, S., Hashiguchi, T., Maruyama, I. (2010) 1,5-Anhydroglucitol attenuates cytokine release and protects mice with type 2 diabetes from inflammatory reactions. *Int J Immunopathol Pharmacol.* **23**, 105-119.

87. Kato, A., Kunimatsu, T., Yamashita, Y., Adachi, I., Takeshita, K., Ishikawa, F. (2013) Protective effects of dietary 1,5-anhydro-D-glucitol as a blood glucose regulator in diabetes and metabolic syndrome. *J Agric Food Chem.* **61**, 611-617.

88. Tong, L., Xiao, X., Li, M., Fang, S., Ma, E., Yu, X., Zhu, Y., Wu, C., Tian, D., Yang, F., Sun, J., Qu, J., Zheng, N., Liao, S., Tai, W., Feng, S., Zhang, L., Li, Y., Wang, L., Han, X., Sun, S., Yang, L., Zhong, H., Zhao, J., Liu, W., Liu, X., Wang, P., Li, L., Zhao, G., Zhang, R., Cheng, G. (2022) A glucose-like metabolite deficient in diabetes inhibits cellular entry

of SARS-CoV-2. *Nat Metab.* 4, 547-558.

89. Ijiri, M., Yoshinaga, K., Kawaguchi, H., Miyazaki, N., Kawaguchi, M., Fujimoto, Y., Matsuo, T., Maruyama, I. (2023) In vivo metabolism of 1,5-anhydro-g-fructose to 1,5-anhydro-d-glucitol. *In Vivo.* **37**, 1022-1027.