A STUDY OF USING CORNCOB, ASPEN SHAVING, DRIED WATER HYACINTH AND BANANA MIDRIB AS BEDDING FOR LABORATORY MICE

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การศึกษาการใช้ซังข้าวโพด ขี้กบไม้สน ผักตบชวาอบแห้ง และก้านใบกล้วยอบแห้ง เป็นวัสดุรองนอนในหนูเมาส์ มีวัตถุประสงค์เพื่อต้องการทดสอบคุณสมบัติทางกายภาพ ได้แก่ การดูดซับของเหลวและความชื้นบนพื้นผิววัสดุรองนอน และศึกษาผลกระทบในทางสรีรวิทยาต่อสุขภาพสัตว์ ค่าแอมโมเนียที่เกิดจากการเลี้ยงและใช้วัสดุรองนอนทั้ง 4 ชนิดในสัตว์ทดลองประเภทหนูเมาส์ การศึกษามี 2 ส่วน ในส่วนที่ 1 ทำการศึกษาคุณสมบัติการดูดซับของเหลวของวัสดุรองนอนทั้ง 4 ชนิดดังกล่าว โดยการทดสอบการดูดซับของเหลวและการทดสอบความชื้นบนพื้นผิววัสดุรองนอน จากผลการศึกษาคุณสมบัติการดูดซับของเหลวของวัสดุรองนอนทั้ง 4 ชนิด พบวัสดุรองนอนชนิดขี้กบไม้สนมีความสามารถในการดูดซับเชิงปริมาตรดีที่สุดอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับวัสดุรองนอนชนิดอื่น แต่ผลการทดสอบความชื้นบนพื้นผิววัสดุรองนอนไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่มการทดลอง ในส่วนที่ 2 ทำการศึกษาในหนูเมาส์ สายพันธุ์ ICR จำนวน 40 ตัว โดยเลี้ยงหนูเมาส์บนวัสดุรองนอนทั้ง 4 ชนิดเป็นระยะเวลา 4 สัปดาห์ แบ่งสัตว์ทดลองเป็นกลุ่มละ 10 ตัว (กรงละ 5 ตัว) เลี้ยงหนูเมาส์ในกรงเลี้ยงสัตว์ทดลองชนิดพลาสติกใส (static micro isolator) มีการเปลี่ยนกรงและวัสดุรองนอนสัปดาห์ละ 1 ครั้ง มีการตรวจสุขภาพสัตว์ทดลอง Grimace scale วัดปริมาณน้ำและอาหารที่กิน วัดระดับแอมโมเนียในกรงเลี้ยงและชั่งน้ำหนักตัวสัตว์ทดลอง เมื่อสิ้นสุดการทดลองทำการเก็บเลือดเพื่อตรวจค่าทางโลหิตวิทยาและค่าเคมีในเลือด และเก็บตัวอย่างชิ้นเนื้อ เพื่อตรวจลักษณะทางพยาธิวิทยาของตับ ไต ฝ่าเท้า และจมูก ผลการศึกษาไม่พบความผิดปกติที่เกี่ยวข้องกับสุขภาพสัตว์ทดลอง พฤติกรรมที่แสดงถึงความเจ็บปวด และไม่พบบาดแผลภายนอก น้ำหนักตัวของสัตว์ทดลองและปริมาณอาหารที่กินไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่มการทดลอง ปริมาณน้ำที่กินมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่มการทดลอง ในส่วนของค่าแอมโมเนียที่เกิดขึ้นในการเลี้ยงพบกรงเลี้ยงสัตว์ทดลองที่ใช้วัสดุรองนอนชนิดขี้กบไม้สน ก้านใบกล้วยอบแห้ง และผักตบชวาอบแห้ง มีค่าเกิน 25 ppm ในวันที่ 3 หลังเปลี่ยนวัสดุรองนอน แต่ในวัสดุรองนอนชนิดซังข้าวโพดมีค่าเกิน 25 ppm ในวันที่ 7 หลังเปลี่ยนวัสดุรองนอน ค่าทางโลหิตวิทยาและค่าเคมีในเลือดของหนูทุกกลุ่มปกติ อ้างอิงจากแหล่งผลิตสัตว์ทดลองและงานวิจัยที่ตีพิมพ์ก่อนหน้า การศึกษาทางพยาธิวิทยาพบรอยโรคการอักเสบในจมูกของหนูที่เลี้ยงในวัสดุรองนอนชนิดขี้กบไม้สน และก้านใบกล้วยอบแห้ง มีความรุนแรงกว่าหนูในกลุ่มที่เลี้ยงบนซังข้าวโพดอย่างมีนัยสำคัญทางสถิติ พยาธิสภาพ ของไต ตับ และฝ่าเท้าของหนู ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติในระหว่างกลุ่มการทดลอง

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Nantaporn Maytayapirom : A STUDY OF USING CORNCOB, ASPEN SHAVING, DRIED WATER HYACINTH AND BANANA MIDRIB AS BEDDING FOR LABORATORY MICE. Advisor: Assoc. Prof. ANUSAK KIJTAWORNRAT, D.V.M., Ph.D. Co-advisor: Asst. Prof. SAIKAEW SUTAYATRAM, D.V.M., Ph.D.

A study of using corncob, aspen shaving, dried water hyacinth and banana midrib as bedding for laboratory mice. The objective of this study was to evaluate the physical characteristics (i.e., absorbency and surface moisture), the physiological effects and intracage ammonia level of 4 types of beddings in laboratory mice. This study was divided into two parts. In the first part, the fluid absorption properties of these four beddings were studied using measurement of liquid absorption and the surface moisture. The results showed that the volumetric absorbency of aspen shaving was significantly higher than other beddings. While, the surface moisture showed no statistically significant difference among beddings. In the second part, 40 ICR mice were randomly assigned to be housed with each bedding for 4 weeks with 10 mice per group (i.e., 5 mice per cage). Mice were housed in static microisolator cage in which the beddings were changed weekly. Animal health, grimace scale, water and food consumptions, intracage ammonia level and animal body weight were evaluated. At the end of the studsy, blood samples were collected for complete blood count and chemistry profile analysis and organs (i.e., liver, kidneys, foot pad, and nasal passage) were harvested for histopathological study. The results revealed that there was no abnormal clinical sign, distress or external lesions in all mice. Body weight gain and food consumptions were not significantly different among groups. Water consumptions were significantly different among groups. Intracage ammonia levels in the aspen shaving, banana midrib and dried water hyacinth groups were higher than 25 ppm within 3 days after beddings were changed, while intracage ammonia levels in corncob groups was increased above 25 ppm in the corncob group at day 7 after changing bedding. The hematology and blood chemistry parameters in everygroups were normal compare with the reference from the animal vendor and previous studies. Histopathological results showed a higher degree of nasal passage inflammation for mice housed in aspen shaving and banana midrib than that of in corncob. The histopathological lesions of kidney, liver and foot pad were similar among groups of beddings.

These results suggest that corncob, aspen shaving, dried water hyacinth and banana midrib may be used as beddings for laboratory mice due to their good absorbency capacity and low physiological effects. The results also indicate that, the bedding should be changed twice per week when using aspen shaving, dried water hyacinth and banana midrib as bedding for mice housing in the static micro isolator cages.

Field of Study:	Animal Physiology	Student's Signature .....
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LISTS OF ABBREVIATION

ADP	Adenosine diphosphate
ALB	Albumin
ALP	*Alkaline phosphatase*
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BASO	Basophils
BUN	*Blood urea nitrogen*
CBC	Complete blood count
Cd	Cadmium
CFU	Colony forming unit
Cl	Chloride
Cr	Creatinine
EO	Eosinophils
EtOH	Ethanol
GLDH	Glutamate dehydrogenase
GLOB	Globulin
H&E	Haematoxylin and Eosin
Hct	Haematocrit
Hgb	Haemoglobin
ISE	Ion-selective electrode
K	Potassium
LDH	*Lactate dehydrogenase*
Lymph	Lymphocytes
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDH	Malate dehydrogenase
Mono	Monocytes
MPN	Most probable number.
MPV	Mean platelet volume
MRM	Murine respiratory mycoplasmas
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
Neu	Neutrophils
PCT	Plateletcrit
PCV	Pack cell volume

PDW	Platelet distribution width
PLT	Platelet count
RBC	Red blood cell
RET	Reticulocyte haemoglobin equivalent
RWD	Red cell distribution width
TAPS	N-Tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid
TOOS	3-(N-Ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid sodium salt
TP	Total protein
TRIS	Tris (Hydroxymethyl) aminomethane
UA	Uric acid
WBC	White blood cell

CHAPTER I

INTRODUCTION

In the rodent laboratory setting, contact bedding management is one of the most important concerns as it is the micro environment that can affect animal health (Carter and Lipman, 2018). Several types of mice bedding are commonly used worldwide such as corncob, aspen wood chip, paper chip and rice hulls. These beddings have been reported to affect animal health and comfort as well as occupational hazard on personnel.

In general, corncob and aspen shaving or chip are commercially available beddings for rodents because they are economy and produce low intracage ammonia (Perkins and Lipman, 1995). However, there are several reports concerning the use of corncob as bedding such as contamination of some substances that could disturb breeding performance (Markaverich et al., 2002), delaying time of vaginal opening (Thigpen et al., 2008) and neuroendocrine function of rodents (Landeros et al., 2012). Moreover, the hard and spherical shape of corncob is uncomfortable for rodents (Ras et al., 2002). Aspen shaving or chip had low contamination rate of tars and resins leading to low effect in physiological or behavioral parameters (Jackson et al., 2015). However, endotoxin and bacterial contaminations during the processing have been reported (Whiteside et al., 2010).

Currently, there are many rodent facilities in Thailand in which corncob and wood shaving or chip are used as rodent beddings. These beddings are expensive and may contribute to research outcome if they are not well preparing or transporting. Thailand is an agricultural country that produces tons of agricultural by-products in which many of them can be used as bedding for rodents.

Water hyacinth (*Eichhornia crassipes*) is aquatic plant that has tremendous absorptive capacity. The water hyacinth has been used for minimizing contaminants in the industrial wastewater (Priya and Selvan, 2017). Recently, dried water hyacinth is locally and commercially available in Thailand as laboratory rodent beddings. However, its effects on animal health and biochemical markers have not been studied in mice.

Banana plants (*Musa balbisiana*) are a perennial fruit crop in which their leaves offer several values such as wrapping the food, blocking the rain, healing burns, etc. Dried banana midrib can be used to make rope, string, and baskets. Due to its unique absorbency property, dried banana midrib may be used as a bedding for rodents. However, no data available to support the use of dried banana midrib as laboratory rodent bedding.

From all of above concerns, this study aims to evaluate the physical characteristics, the physiological effects and intracage ammonia level of these four types of beddings (corncob, aspen wood shaving, dried water hyacinth, and banana midrib) in laboratory mice. We hypothesized that all of these four beddings have equally absorbency capacity and the surface moisture capacity, with no adverse effects on mice when used as bedding and produce intracage ammonia level within normal limit.

CHAPTER II

REVIEWS OF LITERATURE

Laboratory rodent bedding

Rodent bedding is an essential part of the husbandry added to most of the small-sized laboratory animals. It serves many important purposes in animal husbandry. For instance, it is used to absorb excessed fluid from drinking water and animal urination and prevent the animals to contact with its feces. Moreover, mice can use it to build a nest (nesting behavior) or a covering burrow to protect from cold environment, thus bedding can facilitate environmental enrichment in mice. In terms of biological function, some bedding can prevent microorganism growth and intracage ammonia buildup (Smith et al., 2004). Thus, using inappropriate bedding could interfere the treatment effect and research result.

Rodent beddings can be divided into two types: contact and noncontact bedding. By meaning, contact bedding is the bedding that have direct contact with animals (i.e., animals can lay on or burrow in the bedding). Noncontact bedding is the material, usually a sheet or a roll of a pan or cage lining putting under a rack or cage to absorb excrete, thus it does not come into physical contact with the animals. Bedding selection should be based on a several important points (Blom et al., 1996). The major factors that should be concerned when choosing bedding material are absorbency capacity, animal comfort, physiological effect especially species-specific effect, facility management and costs (Kraft, 1980; Wirth, 1983). First, the appropriate bedding should have a good absorbency capacity in order to control surface moisture and ammonia accumulation, both of which can be harmful to the animal health. Second, the bedding texture should be soft and non-sharping to ensure animal comfort. Third, the bedding should not contain any substance that can alter the physiological function of animals. In addition, species-specific effect should be concern especially in the research setting as it can significantly influence study result and inter-species application. For facility management, the bedding should be easy to be stored, prepared, changed and disposed. In addition, it should not create occupational hazard. Lastly, the bedding should be affordable and available in large quality.

Nowadays, most of the animal facilities use corncob and aspen chip as the standard beddings. These beddings had advantages such as commercially available, quality control production, well absorbency and low impact on physiological effects. However, some studies found that both of them contained some substances that could alter physiological parameters in rodents (Markaverich et al., 2002). Also, in some areas, the values of these bedding are quite expensive because of the high transportation cost. Therefore, some local agricultural products such as water hyacinth had been developed to be used as the rodent bedding by some laboratory animal facilities in Thailand. Although, these local agricultural products are inexpensive, the scientific information in terms of physiological effect in mice has not been elucidated. The bedding information in detailed regarding to the production and scientific reports of corncob, water hyacinth, aspen chip and banana midrib is as followed.

1. Aspen shaving

Aspen shaving is produced from aspen wood (*Populus adenopoda*) which is one of the hard woods growing in high mountains in cold regions. Aspen wood has been used as the firewood, house structure and rodent bedding. Aspen wood bedding production is start from shaving or chipped the aspen wood stem into paper thin slices (aspen shaving) or small chips (aspen chip), respectively. With the comfortable texture of aspen bedding, most of the rodents prefer to rest on the aspen chip compare to corncob (Krohn and Hansen, 2008). Also, aspen chip had the higher absorption capacity than corncob (Mason and Burn, 2004). Both of aspen bedding types are the popular beddings because aspen wood has low contamination rate of many substances, especially tars and resins. Moreover, it showed low liver enzymes and other physiological or behavioral effects in mice compared with other hard woods (Jackson et al., 2015). For example, previous study found that the mortality rates of rat pups that were born in shredded aspen and corncob were lower than in cedar shavings (Burkhart and Robinson, 1978). Also, other hard wood beddings such as pine shavings and eucalyptus chip showed harmful effects on breeding achievement (Wilke and Potgieter, 1997). Furthermore, aspen chip contained some sawdust and may be contaminated with the wood preservatives such as penta-chloric phenol, tributylic tin compounds and chromium and copper salts that can affect the physiological functions of rodents (Wirth, 1983). Moreover, hardwood sawdust might increase risk of nasal cancer (Leclerc et al., 1994) and lung tumor

(Witschi et al., 1993) in humans. Also, high level of endotoxin that could cause respiratory syndromes and immunologic responses in rodents was reported (Whiteside et al., 2010).

2. Banana midrib

Banana midrib is one of the waste products from the banana industry that can be used as fertilizer. Banana is one of the primitive cultivated trees in south-east Asia and the pacific island (Swangpol, 2003). Banana has a wide variety of species in both naturally and agriculturally grown, but *Musa balbisiana* is the banana specie that is the easiest to be cultivated in Thailand. Most of it found in the northeast and the south parts of Thailand. Approximately, Thailand has 16,376 rai of planted bananas, in which 15,328 rai was already yielded. Sukhothai province had the largest growing banana areas and it supplied banana leaf and 40,988,916 kilograms of banana. Therefore, the banana midrib can be considered as the significant waste product in Thailand. However, banana production is not stable because it depends on the weather. Banana plant fibers compose of cellulose and hemicellulose that can be used in polymer industries (Deepa et al., 2011) and in the handcraft products. Moreover, banana fruit contains high vitamin B5 and vitamin C; therefore, it can be used in cosmetic industry as well. Because not all of the banana industries are pesticide control, the banana midrib can be contaminated with several toxins including heavy metals and pesticides (Lin et al., 2010). However, dried banana midrib has not been studied as a bedding for rodents.

3. Corncob

Corncob is the core of the corn (*Zea mays*) that is already removed the seed, dried and then grinded into smaller pieces. It has very hard and firm texture. Nowadays, corncob is the most popular choice for rodent bedding due to it contains low level of dust resulting in low risk of allergic reaction of the workers. It also has benefit in low level of intracage ammonia buildup leading to less mucosal irritation and longer cage-changing interval (Wirth, 1983; Ras et al., 2002; Krohn and Hansen, 2008). However, the hard and spherical shape of corncob make it uncomfortable for rodent (Ras et al., 2002). Thus most of the rodents do not prefer to rest on corncob (Krohn and Hansen, 2008). In fact, it showed the significant influence in slow wave

sleep in rats (Leys et al., 2012). Moreover, the contamination of tetrahydrofurandiols, the linoleic acid derivatives with estrogen properties that could disturb breeding performance (Markaverich et al., 2002) and neuroendocrine function, was reported in rodents (Landeros et al., 2012). Also, In some batches of commercial corncob, high level of endotoxin causing respiratory syndromes and immunologic responses was also reported in rodents (Whiteside et al., 2010).

4. Water hyacinth

Water hyacinth (*Hibiscus cannabinus*) is an aquatic plant naturally growing all year round in basin field around tropical and subtropical regions (Zhang et al., 2010). This aquatic plant can spread rapidly and cover the pond surface in a short time (Malik, 2007). However, it has some important roles in the ecological and socio-economical aspects (Villamagna and Murphy, 2010). Water hyacinth has an ecological benefit in its high water pollutant absorption capacity, thus it is usually contaminated with several substances found in that water such as heavy metals (Tiwari et al., 2007) and organic materials (Zimmels et al., 2007). In Thailand, some animal facilities have been developed dried water hyacinth and use it as the rodent bedding but there is no control study to evaluate the quality or physiological effects of these bedding in rodents.

Physical characteristic evaluation for rodent bedding

As mentioned before, important physical characteristics of rodent beddings that should be concerned in laboratory setting are absorbency capacity and surface moisture. They can heavily affect animal well-being in terms of both animal comfort and health. Thus, these two parameters have been evaluated in most of the commercial and local developed beddings to examine the feasible of using as rodent bedding in both of the animal welfare and facility management.

1. Absorbency measurement method for rodent bedding

The primary purpose of the rodent beddings is to absorb excessed humidity from drinking water, urine, and feces. Fully absorbed beddings may reduce its ability in the gas absorption, most importantly in harmful gases such as ammonia and carbon dioxide. Also, high moisture beddings allow bacterial over growth leading to higher risk of bacterial toxin problems (Raynor et al., 1983). When the ammonia level

reaches to its toxic level, it can damage the respiratory system structures and functions, causing rhinitis, otitis media, tracheitis, and pneumonia (including bronchiectasis) similar with the lesions found in murine respiratory mycoplasmas (MRM) (Broderson et al., 1976). Hyperplasia of the tracheal epithelium was presented when rat exposed to the high ammonia for 4 days. However, highly absorbent beddings are not recommened for animals with ringtail or dry skin conditions as it could aggravate the dry skin irritation (Gamble and Clough, 1976).

For bedding absorbency characteristic, the result of previous study show that corncob had the best absorbency capacity compared with aspen chip, loose pulp bedding, and reclaimed wood pulp (Mason and Burn, 2004). Nonetheless, when compared absorbency capacity of corncob, wood chips, and para-rubber, corncob had the lowest absorption (Kengkoom et al., 2008). The result from the another study on the absorbency of corncob, recycle wood pulp, and rice hulls showed that corncob and recycle wood pulp had significantly higher absorption capacity than rice hulls (Carbone et al., 2016).

2. Surface moisture measurement method for rodent bedding

Apart from absorbing excessed moisture, the bedding should serve animals as a comfortable surface that animals can rest on, an insulator that can protect animals from temperature fluctuation, and an environmental enrichment that the animals can exhibit nesting and digging behaviors (Wolfensohn and Lloyd, 2008). The commonly used method for surface moisture measurement in bedding is cobalt chloride test paper (Carbone et al., 2016). Also, the present of wet bedding area in the large part of the floor clearly affects the animal health and welfare. As contacting with hard floor with high moisture on the surface for a long period of time could injure animal foot pad (Weaver and Meijerhof, 1991). The high moisture contributes to high relative humidity that can affect the animal health and welfare. In previous study, 12-18% of relative humidity with high airflow could significantly increase ocular irritation in rodents (Chen et al., 2008b). Also, 15-30% of relative humidity could delay puberty in female mice (Drickamer, 1990). Likewise, the increase humidity up to 40% could increase the incidence of ringtail in rodents (Crippa et al., 2000). For surface moisture study of beddings, dried rice hull bedding had more moisture on the surface than the corncob and recycled wood pulp beddings (Carbone et al., 2016).

Contamination examination for rodent bedding

Several substances and organisms can be contaminated in bedding during natural and facility processes. Previous studied reported that aspen wood could be contaminated with tar, resin (Jackson et al., 2015), saw dust, wood preservative, chromium, copper salt (Wirth, 1983), and endotoxin (Whiteside et al., 2010). Corn could also be contaminated with endotoxins (Whiteside et al., 2010), heavy metals (e.g., cadmium, copper, and lead) (Wang et al., 2017), and several pesticides (Panuwet et al., 2012). Therefore, all of the batches of bedding should be tested for important contaminations especially for serious pathogen organism, heavy metals, pesticides, and some bioactive substances. In general, laboratory bedding materials should be tested for the contamination such as microbiology, pesticide toxin, heavy metal, and aflatoxin. Bacterial endotoxin from the outer cell wall of coliform could be contaminated in environments (Braun-Fahrländer et al., 2002). Endotoxins are hazard for not only the laboratory animals but also the personels (Kaliste et al., 2004). Previous study in mice showed that corn dust from corncob bedding was also contaminated with endotoxin. The corn dust could induce lung inflammation in mice (Jagielo et al., 1996). There were many rodent beddings that could be contaminated with several microbiological substances. Aspen wood bedding had a report of several contaminations including endotoxins and (1,3)-β-D-glucans (Ewaldsson et al., 2002). The sources of (1,3)-β-D-glucans include cell walls of fungi, yeasts, algae, some bacteria, and plants (Roslansky and Novitsky, 1991). In previous study, aspen wood bedding contained more endotoxins than corn and paper beddings (Whiteside et al., 2010). The previous study showed that wood bedding had twice concentrations of endotoxins and (1,3)-β-D-glucans than paper bedding (Ewaldsson et al., 2002). Rodent housed on paper bedding and exposed to aerosolized endotoxin (4 ng/animal daily) and (1,3)-β-D-glucans (1.6 and 16 ng/animal daily) showed inflammatory lung reactions and pulmonary lesions (Ewaldsson et al., 2002). Pesticides are used worldwide for insect control. Most of pesticides are harmful to both animals and humans. Previous rodent study showed that the clinical signs, such as acute poisoning symptoms of micturation, restlessness, pupil constriction, respiratory distress, and convulsion, were developed after the animals exposed to the organophosphorus pesticide (Chedi and Aliyu, 2010). For heavy metals, contaminated substatnces could release hevy metals into surrounding environtment constantly. When animals ingeste and/or inhale hevy metal contaminated particles, it

can cause accumulation of hevy metals in blood and other tissues and may lead to hevy metal poisioning (McDowell, 2003). The clinical signs of some important heavy metals were showed in the table 1.

Table 1. The clinical signs of heavy metal poisoning in animals.

Heavy metals	Clinical signs	References
Arsenic	Abdominal ache, onerwhelming gait, severe weakness, shaking, salivation, vomiting, diarrhea, abstain, weak pulse, fatigue, rumen atony, normal to subnormal temperature, collapse, and death	Selby et al., 1977
Cadmium	Reduction in growth and weight gain Reduced food intake, anemia, enlargement of joints, inflammation of liver parenchyma, renal adjustment, undevelopment of testes, testicular necrosis, and abortion	McDowell, 2003 Habeebu et al., 1998 Djukić-Ćosić et al., 2008 Newairy et al., 2007
Lead	Head-pressing, seizures, agitation, and hyporexia	Palumbo et al., 2010
Mercury	Prolongation of the estrous cycle	Baranski and Szymczyk, 1973

Physiological effect evaluation for rodent bedding

Physiological effects of beddings in laboratory rodents can be measured in several aspects. Animal's appearance, activity, appetite, and body weight can provide basic information of bedding effects on the animal general health. This health observation is quite inexpensive and non-invasive. Thus, daily health check by veterinarians or animal caretakers is crucial and mandatory in standardized laboratory facility. In more specific for bedding evaluation, intracage ammonia level, blood analysis and specific tissue injury or pathological examination should also be included. On the other hand, most of these specific parameters are expensive and invasive in nature. Althrough, not all of these parameters are re-evaluated after the

well-controlled study is conducted. Nevertheless, quality of beddings may vary depended on the plant cultivation and bedding production.

1. Daily health monitoring

The morbidity signs such as external lesions, quiet attitude, ruffled fur, hunched posture, squinted eyes, and porphyrin stained have been known to be associated with abnormal health status, pain, or stress in mice (Burkholder et al., 2012). Therefore, all of the laboratory animal guidelines recommend that veterinarians or experienced animal caretakers should perform daily health observation in all animals individually, especially after starting the study treatment (NRC, 2011). In the effects of beddings, rodents that were kept in the cages with either corncob, rice hull, recycled wood, or pine shaving as a bedding did not show any of the pain signs during 30-day of the study period (Carbone et al., 2016). Another study showed that the mice housed on reclaimed wood pulp, corncob, aspen wood chip, and recycled newspaper were not show any of clinical health problems during daily health checks (Ferrecchia et al., 2014).

2. Weighting monitoring

Animal weight should be monitored weekly to ensure the sufficient food and water consumptions that can be reduced dramatically during severe illness (NRC, 2011). The body weight is also important to calculate medications or test substances, as well as to evaluate the treatment outcomes. For animal humane point, the 10% reduction of body weight is used for considering as the important moribund signs of the study humane endpoint and the animals should be euthanized at this point (NRC, 2011). Furthermore, none of the commonly used or local produced rodent beddings causes serious weight reduction in adult rodents from long-term study (Burn et al., 2006). Another study showed that the rodent housed on wood shavings, perlite, and corncob beddings were not show any effect on the body weight (Yildirim et al., 2017). The mice housed on synthetic wood chip bedding had significant weight loss problem compared with the standard woodchip bedding (Bellin et al., 2019). The body weight of rats housed on spelt was significantly lower than the other rats housed on either aspen wood chips, or corncob bedding (Vogt et al., 2021).

3. Intracage ammonia levels

Intracage ammonia concentration is very important environmental factor, especially in closed-system cage. Despite of the lack of the highest limit of ammonia level in rodent cages, intracage ammonia level at 25 ppm has been purposed as a safe cut off in many rodent guidelines (Reeb-Whitaker et al., 2001).

Ammonia accumulation is affected by several factors including rate of ammonia production that showed variation among animal strains (Smith et al., 2004), cage system or ventilation (Smith et al., 2004; Ferrecchia et al., 2014), and bedding absorbency or characteristics (Wilke and Potgieter, 1997; Smith et al., 2004; Ferrecchia et al., 2014). For animal strain, some diabetic strains of mice and rats produced more urine than their non-diabetic counterparts (Homma et al., 2002). In cage aspect, ammonia will develop and accumulate faster, in the cases that the animal cages are not previously cleaned up properly, over-crowed with animals or poor ventilated, also in the cases that the animals are raised in high water loaded cages or in high ambient temperature and/or humidity that suitable for bacterial growth (Gamble and Clough, 1976). For different in cage system, the individually ventilated cages (IVCs) with a larger amount of bedding could reduce intracage ammonia accumulation (Rosenbaum et al., 2009). The bedding characteristics could alter its absorbency capacity (Mason and Burn, 2004), and might alter its preparation and analysis (Domer et al., 2012).

In previous study, corncob, rice hulls, recycled wood, and pine shavings did not develop intracage ammonia level more than the cut off limit at 25 ppm measured by a multigas analyzer (MultiRAE IR, RAE Systems, San Jose, CA, USA) during the 30-day study period (Carbone et al., 2016). In another study, intracage ammonia levels were compared between static and IVC housing system containing C57BL/6 mice and among 4 types of beddings (i.e., irradiated corncob, reclaimed wood pulp, aspen wood chips, and recycled newspaper) (Ferrecchia et al., 2014). The results indicated that the reclaimed wood pulp bedding increased intracage ammonia level more than the cut off level on day 7 of static cage system and on 2 weeks of IVC cage system. While, the intracage ammonia levels in other beddings did

not reach to the cut off level. Another published intracage ammonia concentration measurement system was a chip measurement system (model 6405300, Dräger Safety, Pittsburgh, PA, USA). This remote system required that the tip of the remote system hose was inserted at the front of the cage to be 2 cm above the bedding surface or at the rodent nose level (Ferrecchia et al., 2014).

4. Biochemical analysis

Blood and biochemical parameters from blood analysis can be used to indicate physiological conditions and pathological effects in humans and animals, since biochemical parameters can be altered by various factors such as physiological condition and environmental stimuli (Quimby, 1999). Common hematological and biochemical parameters measured from blood analysis in rodents were red blood cell, hemoglobin, packed cell volume (PCV), white blood cell count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (Cr), albumin, uric acid, and total protein.

Red blood cell, hemoglobin, and packed cell volume are used to indicate bone marrow function responded to erythropoiesis factors and red blood cell lost from hemorrhage or hemolysis. While, white blood cell count and differential white blood cell count can reflect body response in general for immune function related with infection, inflammation, tissue injury, and immunomodulatory substances.

Important blood chemical parameters are AST and ALT for liver status, albumin for liver function and BUN and Cr for kidney function. AST is actually found in all tissues; however, it shows high concentration in the liver, heart and skeletal muscles. ALT expresses in both cytosolic and mitochondrial forms in the liver (Reitman and Frankel, 1957). Moreover, it also found in high concentration in bone. Nevertheless, increase of AST and ALT usually indicate hepatic injury from inflammation or bile problems. In terms of hematological and biochemical effects of beddings in rodent study, no significant difference in hematological and serum biochemical parameters were seen between male and female mice housed on fresh bedding or recycled bedding from softwood spruce (Miyamoto et al., 2009). However, the results from previous studies found some effects of beddings on biochemical parameters. For example, male and female rats that were housed on wood shaving had higher ALT level than those housed on wheat straw. Also, both male and female mice that were housed on rice straw developed increased AST and ALT

concentrations. While mice housed on wheat straw bedding had lower AST and ALT levels compared with those housed on rice straw and pine wood shaving (Mohamed et al., 2018). Albumin is composed by hepatocytes then it is catabolized in numerous tissues where it is taken up by cellular pinocytosis. The albumin composing amino acids can be utilized by these cells (Grant, 1987). Nonetheless, albumin metabolism can be altered by nutritional status, liver function, and protein losing problems. The urea cycle is related to both ammonia (NH_3) production from protein metabolism in various cell and urea [$(NH_2)_2CO$] production from ammonia by hepatocytes. This cycle appears in ureotelic structure. The urea cycle is important in turning highly toxic ammonia to less toxic urea that can be excreted by urination (Fawcett and Scott, 1960). Besides, uric acid is a chemical compound of metabolized purine nucleotides. Purine is an amino acid normally composed in all parts of the body and are found in some foods and drinks (Grant, 1987). Uric acid is also excreted by urination. As each of these biochemical parameters provide diffent information, investigation of physiological effects induced by beddings on vital organ function and pathological stage should be performed using multiple biochemical parameters together with histopathological examination of suspected organs.

5. Histopathological analysis

Histopathological examination is an important tool to understand the pathophysiology and the extens of impact created from both internal and external stimuli. It is also a gold standard for toxicology study in most laboratory research. Therefore, histopathological study of important organs should be performed before selecting new bedding to reduce the interfrance of bedding on the outcomes of the reasech. Most common organs that have been elvaluated for histopathological study were nasal tissue (Carbone et al., 2016), liver, kidney, and integument system including skin and foot pad (Yildirim et al., 2017).

I. Nasal injury

As previous mention in the intracage ammonia effects on rodent respiratory tract topic that exposure to high concentration of ammonia could injur nasal passage. Nasal epithelium from various locations have been histopathologicaly examined to determine the severity of upper respiratory tract injury in rodents. For example, the intracage ammonia level of more than 25 ppm in the static cage using recycle wood pulp as a bedding could induce the mice to develop multifocal depletion of cilia, variable multifocal submucosal edema, generalized inflammatory

cell infiltrates with a dominance of neutrophils, generalized epithelial necrosis of the turbinate and septal surfaces, hemorrhage, and congestion (Ferrecchia et al., 2014). Moreover, nasal lesions caused by trauma (e.g., olfactory and respiratory epithelial atrophy, degeneration or necrosis, respiratory epithelial hyperplasia and squamous metaplasia, suppurative inflammation, and turbinate lysis) were reported in male and female CD1 mice housed in static cages at 7 days after cage change, where the ammonia levels had increased to an mean of 100 ppm and 64 ppm in static trio and pair cages, respectively (Carpenter et al., 2020).

II. Kidney injury

Various toxic agents could cause nephrotoxic effects leading to histopathological alterations and acute tubular injury such as ischemic or tubulorrhetic acute tubular injury. For instance, marked discoloration of the renal parenchyma in goat following a hemolytic crisis was reported in chronic copper poisoning (Cianciolo and Mohr, 2015). Likewise, renal histopathological findings of cadmium (Cd) and/or ethanol (EtOH) toxicity in rat model were mononuclear cells infiltration combined with degeneration of tubular epithelia, dilation of renal glomeruli, hypertrophy of epithelial cells of renal tubules and hyperaemia of medullary and cortical parts with mononuclear cell infiltration (Brzoska et al., 2003).

III. Chronic hepatotoxicity

Histopathological findings of liver are varied depending on the causes, exposure time and concentration, as well as the timeline or pathophysiological stages. Plant-derived and environmental toxins such as aflatoxin can cause diffused hemorrhage and excessive hepatic necrosis in gross autopsy in dogs, rats, ducks, guinea pigs, and claves. The liver might be normal or showed some lesions including colorlessness or bile staining in appearance, firmed in texture, enlargement and fine nodular regenerative hyperplasia. Histologically, affected liver presented significant hypertrophy of hepatocytes and their nuclei with focal necrosis or apoptosis. Fatty change found in damaged livers was vary in duration and location. Also, bile pigments expanded in canaliculi and hepatocytes, especially in severe cases. In cases of chronic hepatitis caused by copper contamination in dogs, liver developed hypoplasia with an accentuated lobular pattern. Chronic hepatitis is usually presented with portal and periportal mononuclear cell inflammation and fibrosis.

Tiny accumulations of pigmented morphages, contained copper and lipofuscin circled by mononuclear inflammatory cells were a major component of excessed copper and hyperplasia nodules and bridging fibrosis developed during the progression. Extremely damaged livers were characterized by architectural distortion, varied from a nodular texture to an end-stage liver (Cullen and Stalker, 2015). Histopathological findings in liver such as increased quantity of nuclear chromatin, necrosis of pycnosis of nuclei, actively acidophilic cytoplasm, increased density of Kupffer cells and mononuclear cell infiltrations were also reported in the rats treated with Cd and/or EtOH study (Brzoska et al., 2003).

IV. Foot pad injury

For foot pad irritation, the surface and content of bedding material can irritate the rodent's foot and can be accurately evaluated with histopathological examination of foot pad. In previous study, acantholysis inflammatory reactions and degeneration of epithelial cells at the skin of foot pad were higher in degree in rats housed in perlite bedding compared with corncob and wood shavings (Yildirim et al., 2017). Also, ulcer and nodular swellings of hind foot were more severe in the rodent housed on the wire-bottom than polycarbonate cages (Peace et al., 2001). In a rabbit study, improvement of husbandry conditions could significantly decrease prevalence of footpad lesion in farmed rabbits (Rosell et al., 2013).

CHAPTER III

MATERIALS AND METHODS

This study was divided into two experiments. The first experiment assessed physical characteristics related with absorbency capacity and surface moisture of all four beddings (aspen shaving, banana midrib, corncob, and dried water hyacinth). Then, the second experiment evaluated physiological effects of these four beddings in mice.

Approval

This study was approved by the Institutional Animal Care and Use Committee of National Laboratory Animal Center, Mahidol University (Protocol No. 17/2563). All animal procedures were performed under the Regulations and Animals for Scientific Purposes Act, B.E. 2558 (A.D. 2015) and followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (NRC, 2011).

Beddings

Corncob (particle size: 1/8 inch, density: 173.37±1.42 g/500 cm^3), dried water hyacinth (particle size: 5 x 10 mm^2, density: 24.80±0.74 g/500 cm^3), and aspen shaving (particle size: 5 x 10 mm^2, density: 32.26±1.34 g/500 cm^3) were supplied by the National Laboratory Animal Center, Mahidol University. Banana midrib (particle size: 10 x 10 mm^2, density: 50.98±1.03 g/500 cm^3) was purchased from Ban Khlong Krachong Community Enterprise (Figure 1).

Figure 1. Types of test beddings; aspen (A), banana midrib (B), corncob (C) and dried water hyacinth (D)

One sample of each bedding was sent to be analyzed for pesticide, aflatoxin, and heavy metal contaminations, as well as microbiological examination by Asia

Medical and Agricultural Laboratory and Research Center, Bangkok, Thailand before used in the experiment. The test results were showed in table 2. The detection ranges of test method were 0.003-0.01 mg/kg for pesticides (carbamate group, organochlorine group, organophosphate group, and pyrethroid group), 0.01-0.7 µg/kg for aflatoxins, 0.001-0.03 mg/kg for heavy metals, and not limited in microbiological examination. To follow the suitable volume and depth for corncob and aspen shaving suggested by Carbone and co-worker in 2016. These beddings were added to individual cage as followed: corncob 500 cm^3 (approximately 1/4 inch depth); water hyacinth, aspen shaving, and banana midrib 1000 cm^3 (approximately 1 inch depth).

Table 2. Pesticide, aflatoxin, heavy metal and microbiological contaminations of four beddings.

Contamination parameters	Aspen shaving	Banana midrib	Corncob	Dried water hyacinth	Acceptable contamination ranges*
Pesticides (mg/kg)	Not detected	Not detected	Not detected	Not detected	0.005-1.0
1. Carbamate group					
2. Organochlorine group					
3. Organophosphate group					
4. Pyrethroid group					
Aflatoxins (µg/kg)	Not detected	Not detected	Not detected	Not detected	10-15
1. Aflatoxins B1					
2. Aflatoxins B2					
3. Aflatoxins G1					
4. Aflatoxins G2					
5. Total Aflatoxin					
Heavy metals (mg/kg)					
1. Arsenic	Not detected	0.04	Not detected	0.29	0.1-0.5
2. Cadmium	0.08	Not detected	Not detected	Not detected	0.05-0.5
3. Lead	Not detected	<0.03	Not detected	0.11	0.1-1
4. Mercury	Not detected	Not detected	Not detected	Not detected	0.001-1
Microbiological					
1. Total plate count (CFU/g)	<10	<10	<10	<10	$1x10^5$
2. Coliforms (MPN/g)	<3.0	<3.0	<3.0	<3.0	
3. Total mold count (CFU/g)	<10	<10	<10	<10	$\leq 1x10^2$
4. Salmonella spp. (CFU/g)	Not detected	Not detected	Not detected	Not detected	<10

*Acceptable contamination ranges from CODEX, 2010. Abbreviations: CFU = colony forming unit, MPN = most probable number.

Animals

Forty of 3 weeks old, male ICR mice *(Mus Musculus)* were purchased from National Laboratory Animal Center, Mahidol University. All mice were tested to confirm to be free of Sialodacryoadenitis virus, Sendai virus, Mouse hepatitis virus, *Mycoplasma pulmonis*, *Clostridium piliforme*, *Salmonella* spp., *Bordetella bronchiseptica, Citrobacter rodentium, Corynebacterium kutscheri, Mycoplasma pulmonis, Pasteurella multocida, Pasteurella pneumotropica, Streptococcus pneumonia, Streptococcus zooepidermicus, Pseudomonas aeruginosa, Staphylococcus aureus, Yersinia pseudotuberculosis, Asterococcus muris, Citrobacter freundii, Serratia marcessens, Aeromonas hydrophila, Corynebacterium bovis, Dermatophyte, Giardia muris, Spironucleus muris, Syphacia* spp., *Eimeria* spp., and Ectoparasites. The animals were housed in polycarbonate shoe box cage with filter cap (CL-4156-W, CLEA Japan, Inc., Japan) maintained at a temperature of 22±3°C with a relative humidity of 60±10%, 10-15 air changes per hour at cage level and a 12:12 h light: dark cycle. They were quarantined and acclimatized for 7 days in the assigned animal room. The food and water were given *ad libitum*. The beddings were changed once a week.

Experiment I: Physical characteristics of beddings

1. Absorbency measurement

A 500 cm^3 of bedding was measured using a volumetric beaker and weighed using two-digit digital scale. This step was repeated 10 times per bedding type, and the averaged weight was calculated. A 500 cm^3 of each bedding that weight equal to the averaged weight was split into 10 equal-weight portions (i.e., 50 cm^3 in volume) before put each bedding portion into a container. Then, 100 mL of saline was poured into each container. All 10 samples of each bedding were left to be saturated for 1 hour. After soaking, each container was emptied into a sieve. The sieve was shaken at 250 rpm for 1 minute by shaking machine to remove unabsorbed saline, and the wet bedding was weighed. The volume of saline absorbed was analyzed by minus the wet bedding weight with the dry bedding weight. Absorbency by volume and absorbency by mass (weight) were analyzed from the bedding sample volume, bedding sample mass and volume of saline absorbed (Carbone et al., 2016).

Data analysis

For bedding absorbency, the volume of saline absorbed was calculated by minus the wet bedding weight with the dry bedding weight. Absorbency by volume and absorbency by mass were calculated using the following formula:

1. The volume of saline absorption

   Wet bedding weight - dry bedding weight
2. Volume absorbency

$$\frac{\text{The volume of saline absorption}}{\text{Volume of bedding}}$$
3. Mass absorbency

$$\frac{\text{The volume of saline absorption}}{\text{Mass of bedding}}$$

2. Surface moisture

These beddings were added to individual cages: corncob was added 500 cm^3 (approximately 1/4 inch depth); Water hyacinth, aspen shaving, and banana midrib were added 1000 cm^3 (approximately 1 inch depth). Municipal water with green color was drawn into a 1-mL syringe and used to make 6 wet areas in each cage, by holding the syringe on the top of the bedding surface and releasing 0.5 mL of liquid per area. The areas of the aliquots were standardized in all cages. Surface moisture at baseline and hourly thereafter for 10 hours was evaluated for 3 cages per bedding. Surface moisture was detected using cobalt chloride test paper (Toyo Roshi Kaisha, Jtd., Japan; Figure 2), in which its color changed when contacts with water. A 2.5-cm strip of test paper was placed on the surface of a wetted area, and a 45-g weight was put on top of the test paper to hold it in touch with the bedding substance. The contact surface of the weight was a flat plastic surface of 2 cm in diameter. After 5 second, the weight and test paper were removed, and the test paper was photographed. The photographs were presented in random order to a blinded observer, who rated them as positive (color change) or negative (no color change) (Carbone et al., 2016).

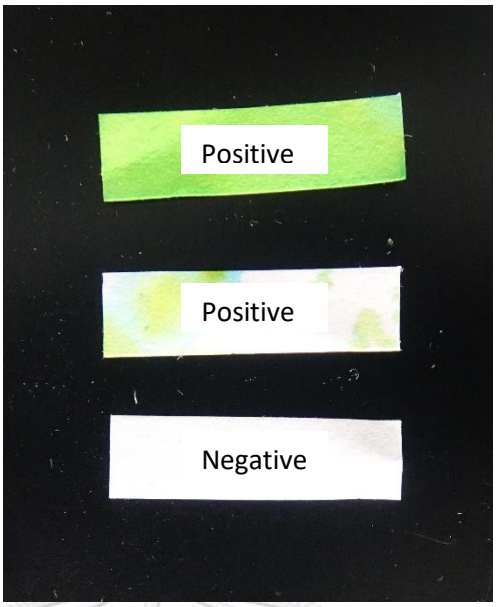

Figure 2. The photographs of cobalt chloride test paper after placed on the surface of a wetted area.

Data analysis

For surface moisture, the test paper photos were given in random order to a blinded observer, the negative and positive results were judged by the blind auditor and were documented in the report.

Statistical analysis

Data were presented as mean±standard deviation (SD). Statistical analyses were performed using Sigma Plot 12.3 software. Normal distribution of continuous data was determined by the Shapiro–Wilk test. Comparing parameters among bedding types were performed using One-way ANOVA with Tukey post hoc test. The non-normal distributed continue data were examined among bedding types by Kruskall-Wallis test. Surface moisture was expressed as odds ratios and 95% confidence intervals. Multiple logistic regression was used to estimate the main effects of bedding type while controlling for time (as a categorical variable) and location within cage. The P value of less than 0.05 was considered as statistical significance.

Experiment II: Physiological effects of beddings

1. Daily health monitoring and weekly weight monitoring.

Male mice were divided into 4 groups (10 mice per group) and 5 mice per cage with one bedding per group. Mice in each group were housed in the assigned type of bedding for 28 days (Figure 3). The cage was filled with beddings to create a depth as followed: corncob 500 cm^3 (approximately 1/4 inch depth); Water hyacinth, aspen shaving, and banana midrib 1000 cm^3 (approximately 1 inch depth) (Carbone et al., 2016). The bedding was changed once a week at 09.00 a.m.-12.00 p.m. While the bedding was changed, the mice were weighted by a calibrated two-digit digital weight scale and weekly body weights were recorded. The body weight gain was calculated by minus the weight of the week 4 by the weight of baseline for all mice. Daily health monitoring and grimace scale were conducted every day at 09.00 a.m.-12.00 p.m. For daily health monitoring, the abnormal sounds, smell and other conditions were also observed and recorded. Abnormal behavior, body contour, and coat changes were inspected in each animal from a distance (Foltz and Ullman-Cullere, 1999). The morbidity signs such as quiet attitude, ruffled fur, hunched posture, squinted eyes, and external lesions were also observed (Carbone et al., 2016). Moreover, the common clinical health conditions of mice such as fight wounds, ear dermatitis, alopecia, tail lesions, and dermatitis were monitored. The general conditions of mice such as diarrhea and anorexia were included in health monitoring (Burkholder et al., 2012). The grimace scale (i.e., orbital tightening, nose bulge, cheek bulge, ear position, and whisker change) was observed and recorded (Langford et al., 2010). The cage appreances including abnormal deposits, and substrate disruption, as well as feed and drinking water amounts were observed. The food and water were weighed using a calibrated two-digit digital weight scale and recorded at the day of bedding change (day 1), then, on day 7 after bedding change. The weights of food and water on day 7 were minuted by the weights of food and water on day 1. The averaged food and water weights over 4 weeks of study period in each bedding were calculated for g/mouse/day for food consumptions and mL/mouse/day for water consumptions. Cages were placed on the housing racks in random order, to reduce viable deviation from light concentration, room airflow and room temperature.

2. Daily intracage ammonia monitoring

Intracage ammonia level was measured daily at 09.00 a.m.-10.00 a.m. by the SMART SENSOR Ammonia Detector (Model AR 8500, Arco electronics ltd., Dongguan, China) consisting of gas-detecting probe and LCD monitor display. According to company product information, the detection range is 0-100 ppm. The basic error is less than ±5% of full scale. The response time and recovery time are less than 60 seconds. The probe was placed in the cage at the level of approximately 2 cm above the bedding surface (the approximate height of a mouse's nose) and approximately 4 cm from the cage wall. This measuring was taken approximately 3 minute and the peak of ammonia level was recorded (Ferrecchia et al., 2014).

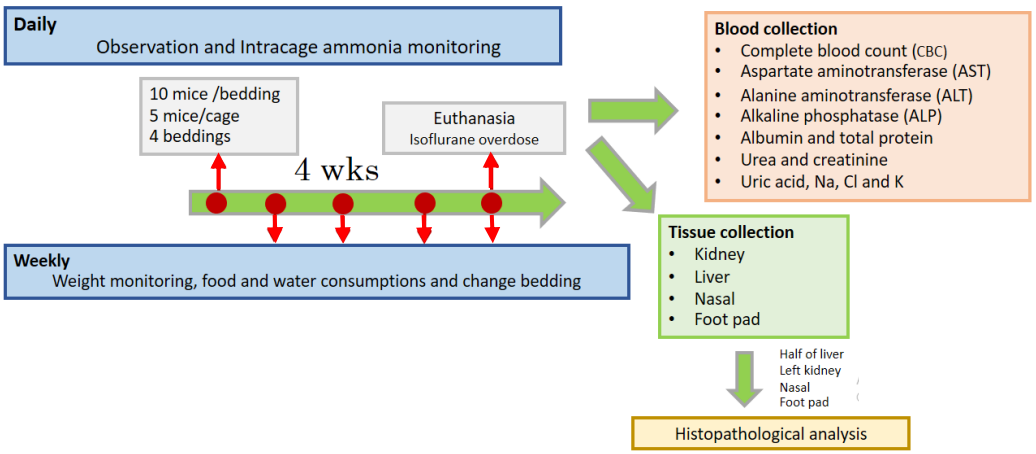

Figure 3. Study timeline

3. Blood analysis

At the end of week 4, all mice were euthanized using overdose isoflurane and 0.6 mL of blood was collected from cardiac puncture. The whole blood (0.2 mL) was transferred into EDTA Eppendorf tube composed of 6.38 g/L sodium chloride, 1.0 g/L boric acid, 0.2 g/L sodium tetraborate and 0.2 g/L EDTA to evaluate complete blood count (CBC) using laser flow cytometry method by Automated Hematology Analyzer (ProCyte Dx™, IDEXX Laboratories, USA). Another 0.4 mL of whole blood was put in Eppendorf tube to separate serum by centrifuged blood at 1008xg for 20 minutes at 4°C and stored at -80°C. Then biochemistry parameters including AST,

ALT, alkaline phosphatase (ALP), albumin, total protein, urea, Cr, uric acid, sodium (Na), chloride (Cl) and potassium (K) were evaluated using Automated Analyzer (Cobas® 4000 analyzer series, Roche Diagnostics GmbH, Germany). The chemical reagents and methods used according to Roche Diagnostics GmbH, Germany for the clinical chemistry analysis were as follow in the table 3.

Table 3. The chemical reagents and methods used for the clinical chemistry analysis

Parameters	Methods	Chemical reagents
Aspartate aminotransferase	The International Federation of Clinical Chemistry	TRIS buffer: 264 mmol/L, pH 7.8 (37 °C); L-aspartate: 792 mmol/L; MDH (microorganism): ≥ 24 µkat/L; LDH (microorganisms): ≥ 48 µkat/L; albumin (bovine): 0.25 %; preservative
Alanine aminotransferase	The International Federation of Clinical Chemistry	TRIS buffer: 224 mmol/L, pH 7.3 (37 °C); L-alanine: 1120 mmol/L; albumin (bovine): 0.25 %; LDH (microorganisms): ≥ 45 µkat/L; stabilizers; preservative
Alkaline phosphatase	Cololorimetric assay	2-amino-2-methyl-1-propanol: 1.724 mol/L; magnesium acetate: 3.83 mmol/L; zinc sulfate: 0.766 mmol/L; N-(2-hydroxyethyl)-ethylenediamine triacetic acid: 3.83 mmol/L
Albumin	Modified bromcresol green binding assay	Citrate buffer: 95 mmol/L, pH 4.1; bromcresol green: 0.66 mmol/L; preservatives, stabilizers
Total protein	Cololorimetric assay	Sodium hydroxide: 400 mmol/L; potassium sodium tartrate: 89 mmol/L
Urea	Enzymatic method	TRIS buffer: 220 mmol/L, pH 8.6; 2-oxoglutarate: 73 mmol/L; NADH: 2.5 mmol/L; ADP: 6.5 mmol/L; urease (jack bean): ≥ 300 µkat/L; GLDH (bovine liver): ≥ 80 µkat/L; preservative; nonreactive stabilizers
Creatinine	Enzymatic, colorimetric method	TAPS buffer: 30 mmol/L, pH 8.1; creatinase (microorganisms): ≥ 332 µkat/L; sarcosine oxidase (microorganisms): ≥ 132 µkat/L; ascorbate oxidase (microorganisms): ≥ 33 µkat/L; catalase (microorganisms): ≥ 1.67 µkat/L; HTIB: 1.2 g/L; detergents; preservative
Uric acid	Enzymatic colorimetric test	Phosphate buffer: 0.05 mol/L, pH 7.8; TOOS: 7 mmol/L; fatty alcohol polyglycol ether: 4.8 %; ascorbate oxidase ≥ 83.5 µkat/L (25 °C); stabilizers; preservative
Sodium Choride Potassium	Ion-selective electrode	ISE reference electrolyte, ISE internal standard, ISE diluent

Abbreviations: TRIS = Tris (Hydroxymethyl) aminomethane, MDH = Malate dehydrogenase, LDH = *Lactate dehydrogenase*, NADH = Nicotinamide adenine dinucleotide, ADP = Adenosine diphosphate, GLDH = Glutamate dehydrogenase, TAPS = N-Tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid, TOOS = 3-(N-Ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid sodium salt, ISE = Ion-selective electrode.

4. Histopathological analysis

Nose, foot pad, liver and kidney were collected from all 40 mice after euthanasia. The stomach, small intestine, large intestine and lower respiratory tracts were cut down and observed for any abnormal sign. The nose, foot pad, half of liver and left kidney were used for histopathological examination. The organs were collected and fixed in 10% buffered formalin solution for 24-48 hours at room temperature and embedded in paraffin. Nasal and foot pad were decalcified before embedded in paraffin. Each histopathological slide was contained each organ from one mouse (i.e., 1 slide/organ and 4 slide/mouse). The longitudinal section of left kidney was cut and arranged at the center of kidney. Cross section of left lobe of liver was used. Nasal sections were made at 1 mm and 4 mm from the tip of nose, and the medial canthus of the eye (Ferrecchia et al., 2014). Longitudinal sections of each foot pad were prepared and cut at the plantar side for evaluation. The thick of paraffin sections from the kidney, liver, nasal and foot pad were 4 μm. All sections were deparaffinized and hydrated, before stained with hematoxylin and eosin dye (H&E).

Data analysis

Histopathological analysis

Histopathological lesions both in cortex and medulla of glomerulus and tubular compartment were examined under a high-power light microscopic examination using Leica DM2000 (Leica microsystems Inc., Buffalo Grove, IL, USA). Grading of tissue lesion was performed by one experienced blinded pathologist. The semiquantitative grading of kidney, liver, nasal, and foot pad in each slide was randomly performed.

For the kidney, the histopathological lesions were evaluated in terms of hypertrophy of epithelial cells of renal tubules, degeneration of tubular epithelium with simultaneous infiltration of mononuclear cells, hyperaemia of medullary and cortical part with mononuclear cell infiltration and dilation of renal glomeruli (Brzoska et al., 2003). For the liver, the histopathological lesions were evaluated in terms of blurred trabecular structure of the lobules, vacuolar degeneration changes, enlarged cell sizes, increased density of nuclear chromatin and very compact nuclear

structure, necrosis of single cells (i.e., pyknosis of nuclei, and strongly acidophilic cytoplasm), increased number of Kupffer cells and sinuses overfilled with blood with mononuclear cell infiltrations (Brzoska et al., 2003). Grading criteria of kidney and liver were scored according to 0 = normal, (i.e., the tissue in each slide was identify to be normal under the conditions of study, age, sex, and strain of the animal involved.); 1 = minimal, (i.e., the tissue in each slide was barely change which seem not more progress.); 2 = mild, (i.e., in hold tissue, the lesion was clearly identified but severity.); 3 = moderate, (i.e., the lesions were dominant, but the severity was increase sharply.); and 4 = marked, (i.e., the quality of alter was as valid as possible.) (Mann et al., 2012).

For the nasal cavity, the histopathological lesions were evaluated in terms of generalized epithelial necrosis of the turbinate and septal surfaces, generalized inflammatory cell infiltrates with a dominance of neutrophils, multifocal depletion of cilia, and variable multifocal submucosal edema, congestion, and hemorrhage (Ferrecchia et al., 2014). The scoring method for each section was based on distribution of each lesion within the nasal cavity, as follows: 0 = no remarkable lesion; 1 = the lesions were involving less than 5% of the epithelium or tissue; 2 = mild, the lesions were involving 5-25% of the epithelium or tissue; 3 = moderate, the lesions were involving 26-50% of the epithelium or tissue; and 4 = marked, the lesions were involving more than 50% of the epithelium or tissue (Carpenter et al., 2020).

For foot pad, the histopathological lesions were evaluated in terms of acantholysis inflammatory reactions and degeneration of epithelial cells (Yildirim et al., 2017). The scores of epithelial degeneration and inflammation were reported as follows: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe (Yildirim et al., 2017). All of the grades were performed base on each parameter. All of the grades of each parameter were summarized before the statistical analysis.

Statistical analysis

Data was presented as mean±standard deviation (SD) and median with range. Statistical analyses were performed using Sigma Plot 12.3 software. Normal distribution was determined by the Shapiro–Wilk test. Physiological parameters among types of bedding were compared using One-way ANOVA with Tukey post hoc

test. If the continuous data is non-normal distributed, the comparison among bedding types is examined by Kruskall–Wallis test. Differences among histopathological analysis of liver, kidney, foot pad and nasal lesions were tested using Kruskall–Wallis test. The P value of less than 0.05 was considered as statistical significance.

CHAPTER IV

RESULTS

Part I

1. Bedding Absorbency

Absorbency by volume was 0.42±0.05 mL/cm^3 for aspen shaving, 0.27±0.02 mL/cm^3 for banana midrib, 0.34±0.04 mL/cm^3 for corncob bedding and 0.17±0.03 mL/cm^3 for dried water hyacinth (Figure 4). Pairwise comparisons for absorbency by volume in beddings were all significant ($P < 0.05$). Aspen shaving had the highest volume absorbency when compare with other beddings. Nevertheless, dried water hyacinth was the least absorbent than other beddings. Absorbency by mass was 6.46±0.85 mL/g for aspen shaving, 2.63±0.23 mL/g for banana midrib, 0.98±0.11 mL/g for corncob bedding, and 3.37±0.59 mL/g for dried water hyacinth (Figure 5).

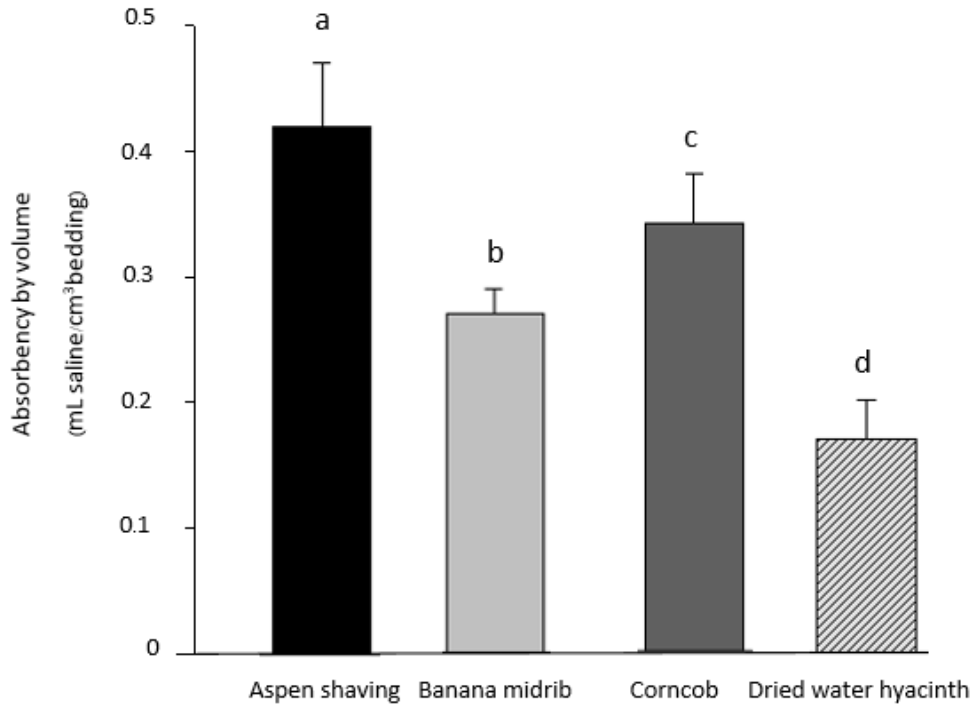

Figure 4. Absorbency by volume of aspen shaving, banana midrib, corncob, and dried water hyacinth (n = 10 for all groups). Data are presented as mean values; error bars, 1 SD. ^{abcd} indicates significant difference ($P < 0.05$) among 4 types of beddings.

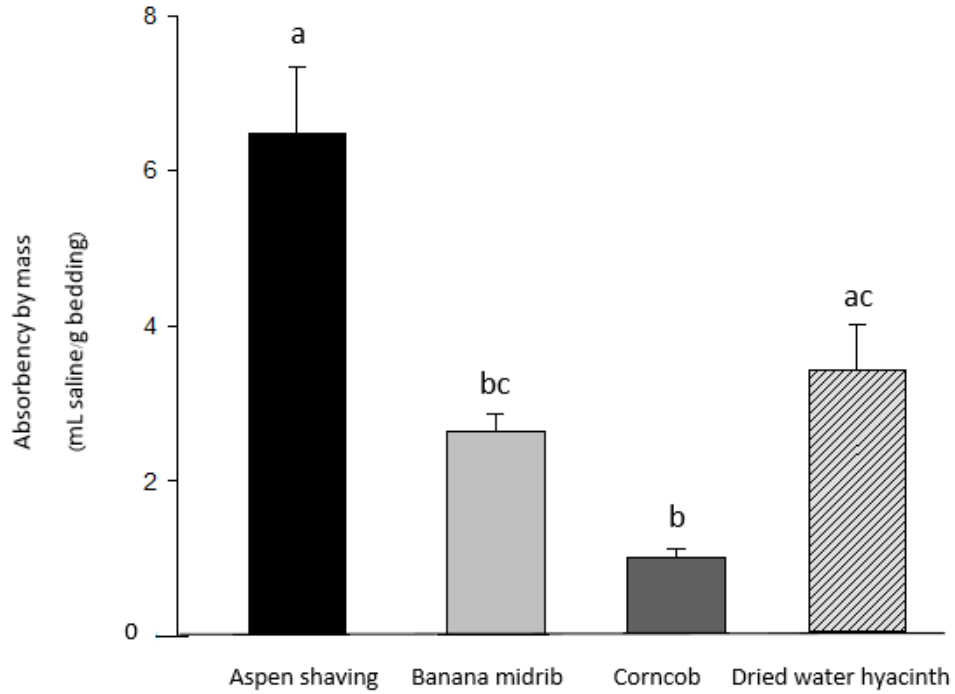

Figure 5. Absorbency by mass of aspen shaving, banana midrib, corncob, and dried water hyacinth (n = 10 for all groups). Data are presented as mean values; error bars, 1 SD. ^{abc} indicates significant difference ($P < 0.05$) among 4 types of beddings.

2. Surface moisture

In terms of surface moisture, multiple logistic regression comparisions among groups of beddings were not significant (odd ratio, 1.132; 95% confidence interval, 0.921 to 1.884; $P < 0.05$). The corncob surfaces were all dried within 1 hour, while the moisture retention time for aspen wood shaving, banana midrib, and water hyacinth were 1, 2, and 5 hours, respectively (Figure 6).

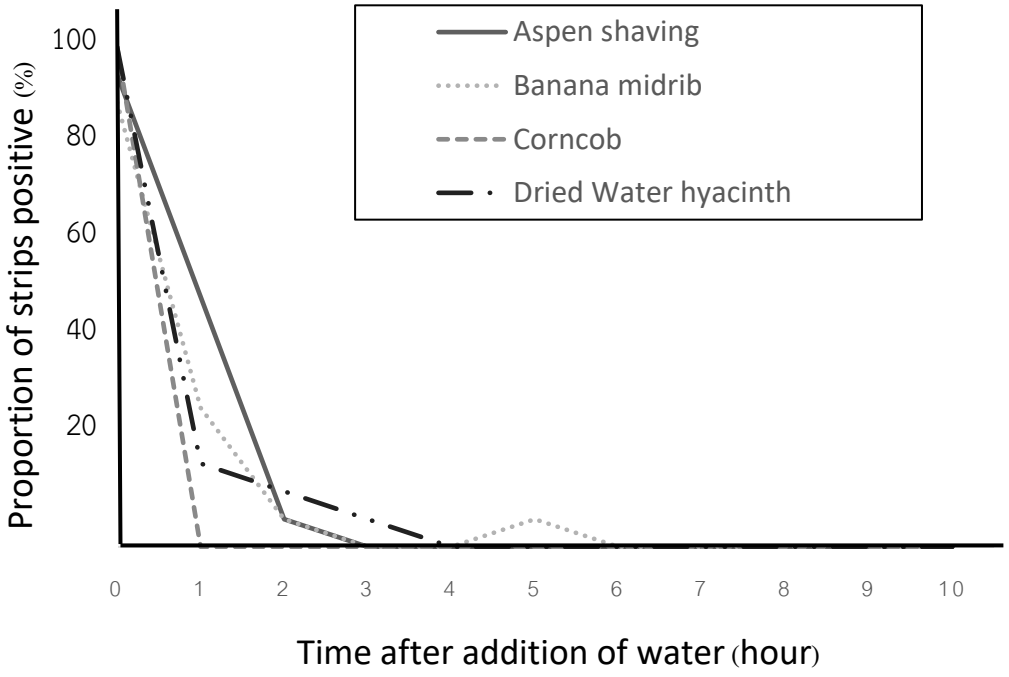

Figure 6. The persistence of surface moisture on aspen shaving, banana midrib, corncob, and dried water hyacinth for 10 hours after application of aliquots of water at 0 hour (n = 3 cage/bedding type with 6 measurement area/cage).

Part II

1. Daily health observations

None of the mice had signs of morbidity (i.e., quiet attitude, ruffled fur, hunched posture, squinted eyes, or external lesions) or external wound on daily observation. For grimace scale, all mice had 0 scale at everyday. The areas of wet bedding at the bottom of cages were observed to be more frequent and larger in the cages with banana midrib and dried water hyacinth than in cages with corncob or aspen shaving bedding.

2. Body weight monitoring

Body weight showed no significant difference among types of beddings. The averaged body weight gains were 14.36±1.88 g for aspen shaving, 13.04±1.87 g for banana midrib, 15.05±2.12 g for corncob and 14.37±1.65 g for dried water hyacinth during 28 days of the study period (Figure 7). The averaged body weights at baseline were 25.34±0.98 g for aspen shaving, 25.70±1.16 g for banana midrib, 24.66±1.07 g for corncob and 25.97±0.71 g for dried water hyacinth (Figure 8).

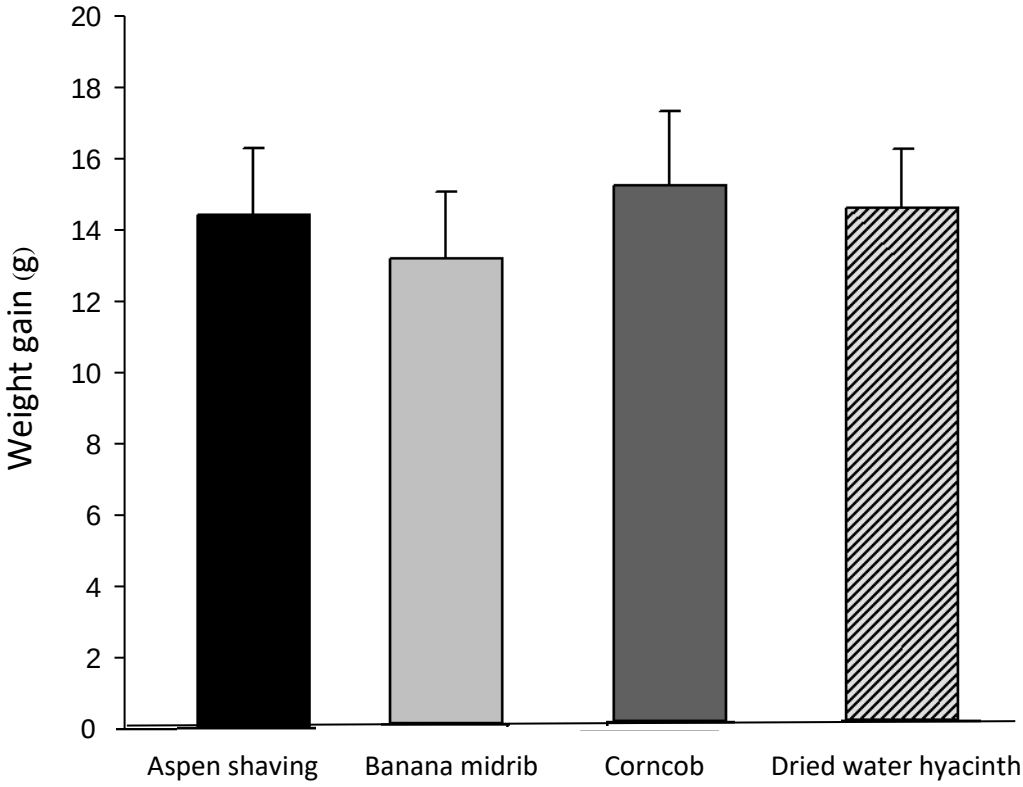

Figure 7. The weight gain per mouse during the 28 days of study period of mice housed in cages with aspen shaving, banana midrib, corncob, and dried water hyacinth (n = 10 per group). Data are presented as mean values; error bars, 1 SD.

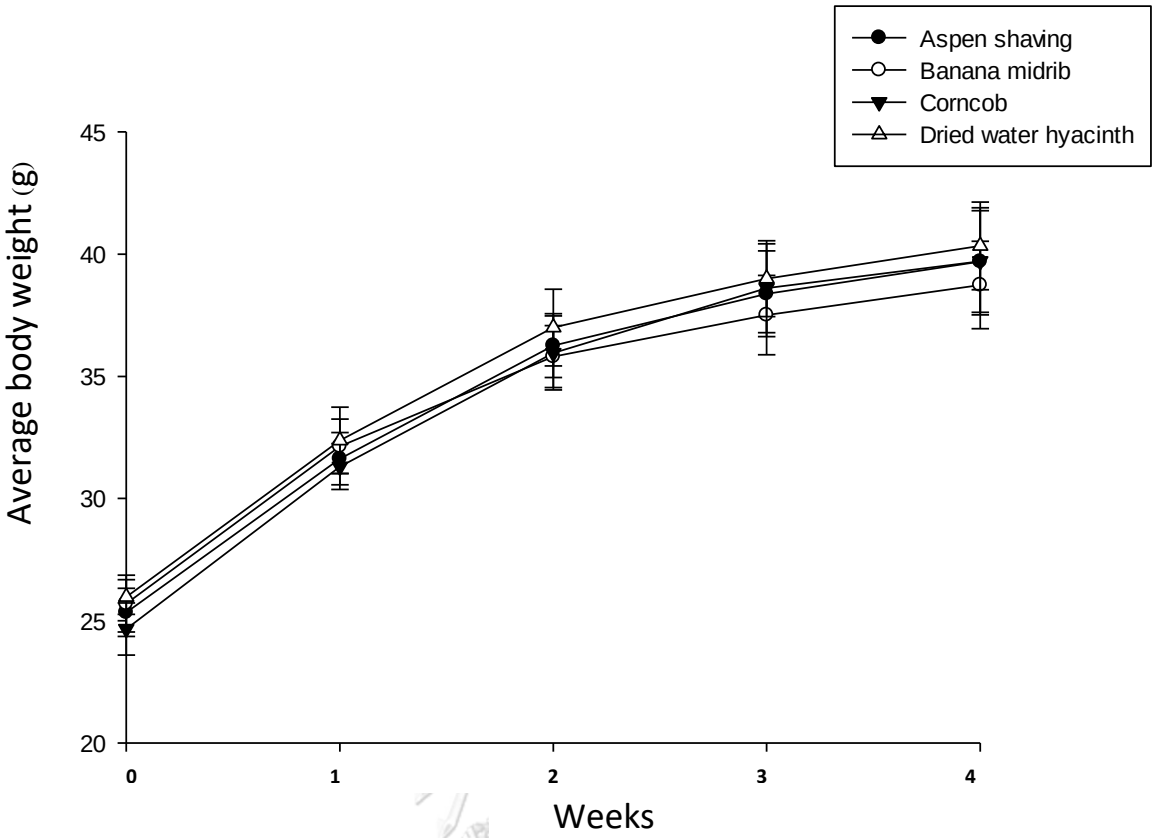

Figure 8. The averaged body weights in each week of mice housed in cages with aspen shaving, banana midrib, corncob, and dried water hyacinth (n = 10 per group). Data are presented as mean±standard deviation (SD).

3. Food and water consumption

Food and water consumption values were presented in Table 4. Food consumption showed no statistical difference, while water consumption was significantly different among types of beddings. The mice housed with dried water hyacinth had the highest water consumption compared with other groups.

Table 4. Averaged food and water consumptions of 40 male mice (10 mice/group).

Parameters	Aspen shaving	Banana midrib	Corncob	Dried water hyacinth
Food consumption (g/mouse/day) (N = 8)	4.88±0.16	4.53±0.32	4.84±0.27	4.73±0.23
Water consumption (mL/mouse/day) (N = 6)	6.09±0.56[a]	7.26±0.91[ab]	6.77±0.80[ab]	8.22±1.53[b]

Data are presented as mean±standard deviation (SD). [ab] indicates in the same rows with difference superscripts were significant difference ($P < 0.05$).

4. Intracage ammonia levels

Intracage ammonia levels were reported as the daily averaged ammonia concentration (ppm) for each bedding type (Table 5). At the third day after changing the bedding, ammonia levels in dried water hyacinth group exceeded the exposure limit of 25 ppm. Moreover, the intracage ammonia levels in aspen shaving and banana midrib groups were higher than 25 ppm at day 4th after changing the bedding. However, intracage ammonia levels in corncob group were higher than 25 ppm at day 7th after changing the bedding. The intracage ammonia levels in aspen shaving, banana midrib and dried water hyacinth groups were significantly higher than corncob group by day 4th after changing the bedding ($P < 0.05$) (Figure 9).

Table 5. The daily averaged intracage ammonia concentrations and the weekly averaged intracage concentration of aspen shaving, banana midrib, corncob, and dried water hyacinth groups (n = 6 for all groups).

Day	Aspen shaving	Banana midrib	Corncob	Dried water hyacinth	Average*
Day 1	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00^{d}
Day 2	0.00 ± 0.00^{b}	0.08 ± 0.21^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.025^{d}
Day 3	21.24 ± 21.29^{b}	19.51 ± 19.60^{bc}	1.34 ± 3.20^{b}	27.11 ± 18.59^{bc}	21.63^{c}
Day 4	$56.23\pm41.55^{a\ x}$	$46.01\pm24.51^{b\ y}$	$3.50\pm4.44^{b\ z}$	$53.81\pm39.87^{ab\ xy}$	41.16^{b}
Day 5	$67.73\pm39.18^{a\ x}$	$58.88\pm27.57^{ab\ x}$	$4.98\pm5.00^{b\ y}$	$61.99\pm33.12^{ab\ x}$	52.01^{b}
Day 6	$87.64\pm30.03^{a\ x}$	$83.11\pm16.73^{a\ x}$	$19.44\pm8.94^{ab\ y}$	$70.74\pm34.68^{a\ x}$	71.40^{a}
Day 7	$81.05\pm36.68^{a\ x}$	$91.54\pm12.93^{a\ x}$	$49.75\pm32.19^{a\ y}$	$84.09\pm29.83^{a\ x}$	84.62^{a}
Average*	53.29^{x}	42.29^{x}	14.0^{y}	45.13^{x}	

Data are presented as mean±standard deviation (SD). ^{xyz} means in the same row with different superscripts are significantly different ($P < 0.05$) (compare different bedding in the same day), ^{abcd} means in the same column with different superscripts are significantly different ($P < 0.05$) (compare the same bedding in different days). *the average data in both row and column indicate the main effect with two way repeated measures ANOVA analysis.

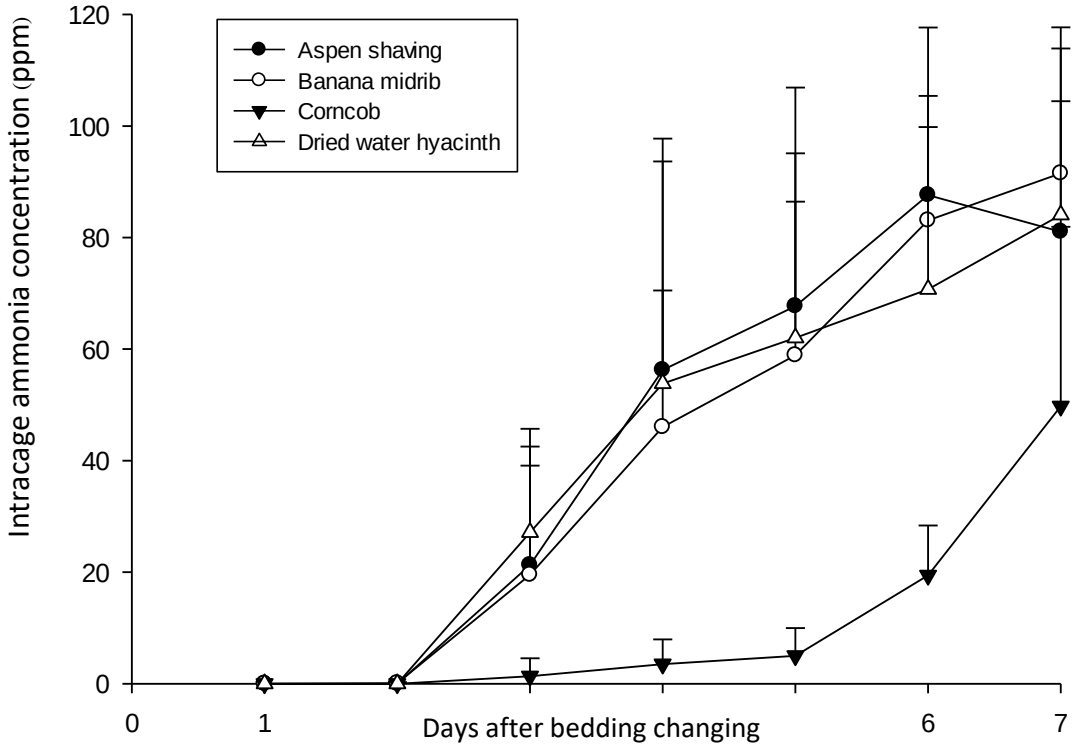

Figure 9. Averaged daily intracage ammonia concentrations in static cages with filter top. Data are presented as mean values; error bars, 1 SD.

Complete blood count and blood chemistry analysis

Hematological and blood chemistry values at 28 days of study in all bedding types were presented in Table 6 and Table 7, respectively. There was no statistical significance of hematological parameters among groups. Also, all hematological parameters were within reference values of male 10 weeks old mice from the animal vendor. Blood chemistry values were within normal ranges. Total protein, uric acid, Na, K, and Cl showed significant differences among groups ($P < 0.05$).

Table 6. Hematological parameters of 40 male mice (10 mice/group).

Hematological parameters	Aspen shaving	Banana midrib	Corncob	Dried water hyacinth	Normal value from NLAC
RBC (10^6/μL)	8.36±0.41	8.50±0.40	8.47±0.48	8.53±0.33	8.49-11.78
HGB (g/dL)	12.99±0.53	13.26±0.89	13.02±0.74	12.94±0.61	12.80-17.40
HCT (%)	40.43±1.94	41.28±2.81	41.19±2.35	41.08±1.53	43.05-61.80
MCV (fL)	48.39±1.87	48.56±1.94	48.64±1.66	48.16±1.50	45.70-66.70
MCH (pg)	15.55±0.51	15.60±0.59	15.37±0.54	15.19±0.62	14.20-17.03
MCHC (g/dL)	32.15±0.72	32.14±0.69	31.63±0.76	31.50±0.65	25.40-32.40
RDW (%)	25.28±1.14	26.00±1.52	25.39±1.62	26.00±1.25	22.20-28.60
RET (10^3/μL)	560.41±66.27	554.18±142.39	535.57±131.48	572.41±95.08	310.70-976.10
PLT (10^3/μL)	918.30±58.48	915.50±43.41	890.13±75.03	941.30±71.90	560-1438
PDW (fL)	7.31±0.33	7.70±0.37	7.20±0.39	7.68±0.44	6.40-9.40
MPV (fL)	6.21±0.24	6.36±0.20	6.21±0.18	6.36±0.22	5.70-7.50
PCT (%)	0.57±0.04	0.58±0.03	0.55±0.04	0.60±0.04	0.41-1.07
WBC (10^3/μL)	3.96±1.34	3.69±1.06	5.20±1.56	5.20±1.17	1.46-7.05
DIFFERENTIAL COUNT (%)					
NEU	16.81±2.49	22.33±5.43	30.44±19.78	21.52±5.72	8.7-52.0
LYMPH	78.77±3.28	71.61±6.29	62.24±22.66	73.36±5.79	39.3-85.2
MONO	1.93±0.74	3.09±1.34	4.89±3.28	3.02±2.33	0.8-19.6
EO	2.31±0.86	2.72±1.13	2.18±0.72	1.98±0.60	0.0-6.0
BASO	0.18±0.21	0.25±0.22	0.30±0.21	0.12±0.11	0.0-2.1

Data are presented as mean±standard deviation (SD). Most of normal reference values from National Laboratory Animal Center (NLAC), Mahidol University. Abbreviations: RBC = red blood cell, HGB = hemoglobin, HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, RWD = red cell distribution width, RET = reticulocyte haemoglobin equivalent, PLT = platelet count, PDW = platelet distribution width, MPV = mean platelet volume, PCT = plateletcrit, WBC = white blood cell, NEU = neutrophils, LYMPH = lymphocytes, MONO = monocytes, EO = eosinophils, BASO = basophils.

Table 7. Biochemistry parameters of 40 male mice (10 mice/group).

Blood chemistry parameters	Aspen shaving	Banana midrib	Corncob	Dried water hyacinth	Normal value from NLAC
ALP (U/L)	144.00±21.39	138.20±14.49	131.70±28.44	138.44±18.88	101-174
ALB (g/dL)	3.71±0.36	3.60±0.30	3.28±0.42	3.66±0.34	3.81-4.57
BUN (mg/dL)	27.92±4.17	28.83±4.28	29.29±3.74	27.72±3.59	22.1-36.4
Cr (mg/dL)	0.10±0.02	0.08±0.01	0.09±0.02	0.09±0.01	0.10-0.16
TP (g/dL)	5.70±0.60^{a}	5.62±0.39^{ab}	5.08±0.43^{b}	5.66±0.50^{a}	5.39-7.06
ALT (U/L)	31.45±8.34	31.51±7.56	37.19±11.11	41.67±18.57	24.9-149.5
AST (U/L)	67.49±11.67	75.45±23.05	77.45±14.58	75.77±21.61	83.7-258.8
UA (mg/dL)	1.78±0.37^{a}	1.68±0.27^{ab}	1.29±0.40^{b}	1.81±0.29^{a}	0.002-12.8*
GLOB (g/dL)	1.99±0.25	2.02±0.16	1.80±0.20	2.00±0.18	2.0-2.52
Na (mmol/L)	160.40±6.17^{ab}	162.70±7.69^{a}	154.00±4.45^{b}	160.50±5.89^{ab}	151-161**
K (mmol/L)	4.96±0.36^{ab}	5.33±0.38^{a}	4.75±0.46^{b}	5.25±0.39^{a}	3.5-5.3***
Cl (mmol/L)	113.50±3.05^{ab}	116.63±4.68^{a}	108.55±4.81^{b}	114.34±3.96^{a}	112-124**

Data are presented as mean±standard deviation (SD). ^{ab} indicates in the same rows with difference superscripts are significant difference ($P < 0.05$). Normal reference values from National Laboratory Animal Center (NLAC), Mahidol University. * reference values from Watanabe et al., 2014. ** reference values from Serfilippi et al., 2003. *** means reference data from Traslavina et al., 2010. Abbreviations: ALP = *alkaline phosphatase*, ALB = albumin, BUN = *blood urea nitrogen*, Cr= creatinine, TP = total protein, ALT = alanine aminotransferase, AST = aspartate aminotransferase, UA = uric acid, GLOB = globulin, Na = sodium, K = potassium, Cl = chloride.

5. Histopathology analysis

All histopathological gradings of nasal, kidney, liver, and foot pad were presented in Table 8. Only the histopathological grading of nasal samples showed statistically significant differences among types of beddings ($P < 0.05$). Also, none of the gross abnormal findings were found in any mouse. No notable lesion was identified in the stomach, small intestine, large intestine, and lower respiratory tracts.

Table 8. Histopathological gradings of 40 male mice (10 mice/group).

Histopathological parameters	Aspen shaving	Banana midrib	Corncob	Dried water hyacinth
Nasal histopathological grading (5 scale)				
Median	3 [a]	2 [a]	0 [b]	2 [ab]
Range	1-4	0-4	0-1	0-4
Number of affected mice	10	9	2	7
Kidney histopathological grading (5 scale)				
Median	0	0.5	1	0.5
Range	0-1	0-1	0-2	0-2
Number of affected mice	4	5	5	5
Liver histopathological grading (5 scale)				
Median	0	0	0	0
Range	0	0	0	0
Number of affected mice	0	0	0	0
Foot pad histopathological grading (5 scale)				
Median	0	0	0	0
Range	0	0-1	0-1	0-1
Number of affected mice	0	1	2	1

Data are presented as median and range with number of affected mice. [ab] indicates in the same rows with difference superscripts are significant difference ($P < 0.05$).

The examples of the nasal passage histopathological findings were showed in Figure 10-12. Coronal sections were made at 1 mm from the tip of the nose, 4 mm from the tip of the nose, and the medial canthus of the eye.

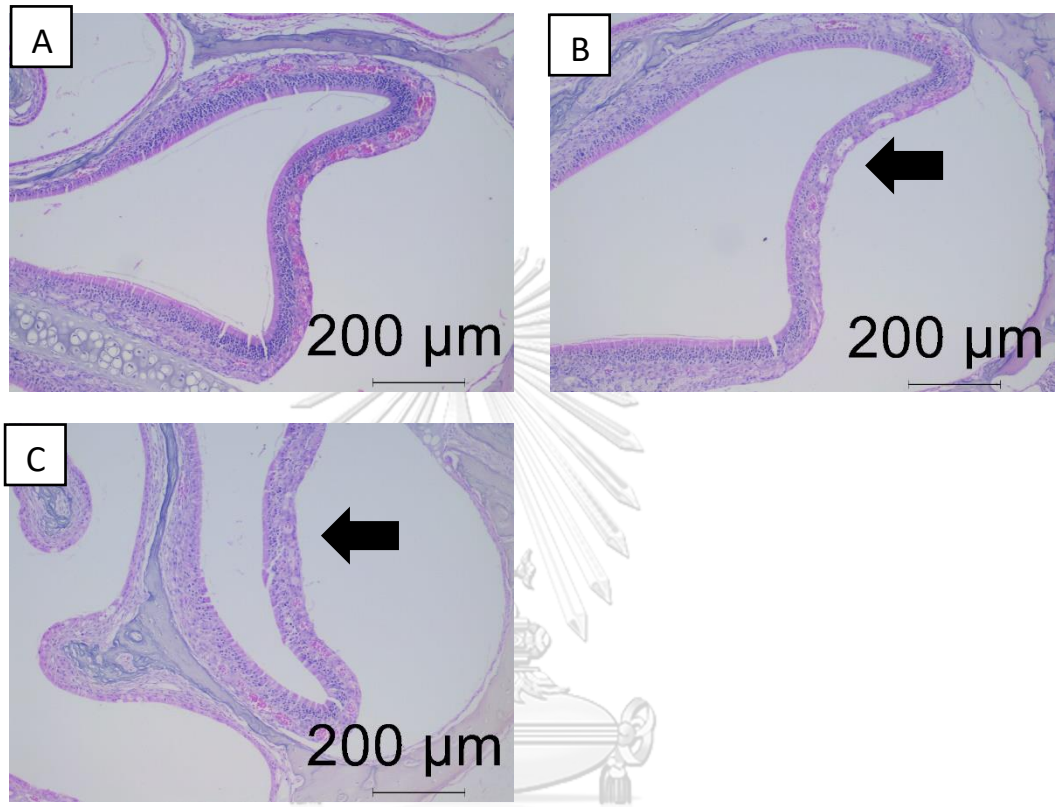

Figure 10. Histopathological changes in nasal sections showed the early necrosis of the olfactory epithelium at 4 mm from the tip of the nose. The example of no remarkable lesion (score 0) from mouse housed in corncob bedding (A). There were areas of the early necrosis of the olfactory epithelium, characterized by pyknotic or karryorhetic nuclei with rare scattered individual neutrophil infiltrates (black arrow) in mice housed in aspen shaving bedding with score 1 (B) and score 2 (C). Scale bar 200 µm. Hematoxylin and eosin (H&E).

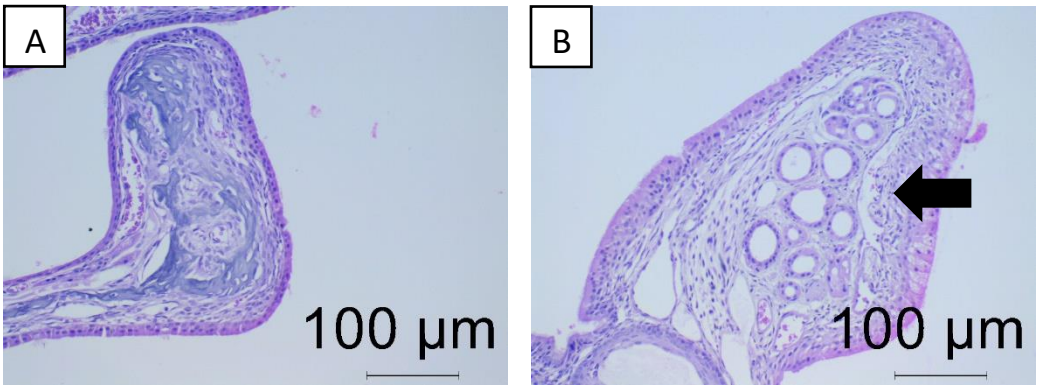

Figure 11. Histopathological changes in nasal sections showed focally cytoplasmic vacuoles of the respiratory epithelium at 4 mm from the tip of the nose. The normal nasal histopathological findings (score 0) from mouse housed in dried water hyacinth bedding (A). The respiratory epithelium had focally cytoplasmic vacuoles (black arrow) in mouse housed in aspen shaving bedding (score 1) (B). Scale bar 100 µm. Hematoxylin and eosin (H&E).

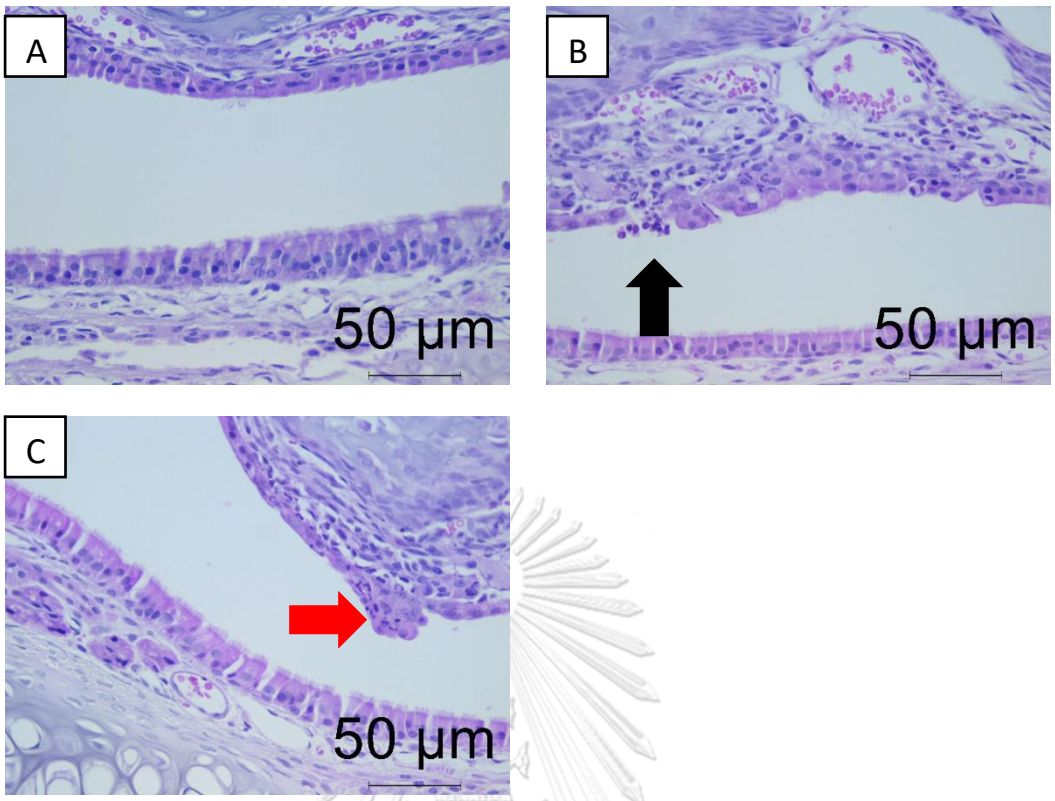

Figure 12. Histopathological changes in nasal sections showed a small focus of erosion/ulceration of the respiratory epithelium at 4 mm from the tip of the nose. The normal nasal epithelium (score 0) was present in mouse housed in dried water hyacinth (A). There was a small focus of erosion/ulceration of the respiratory epithelium with few neutrophil infiltrations over the affected epithelium (black arrow) (score 1) in mouse housed in aspen shaving bedding (B). A focal hyperplasia with neutrophils scattered of the respiratory epithelium (red arrow) (score 1) was presented in mouse housed in aspen shaving bedding (C). Scale bar 50 µm. Hematoxylin and eosin (H&E).

Selected renal histopathological alterations found in all groups were showed in Figure 13.

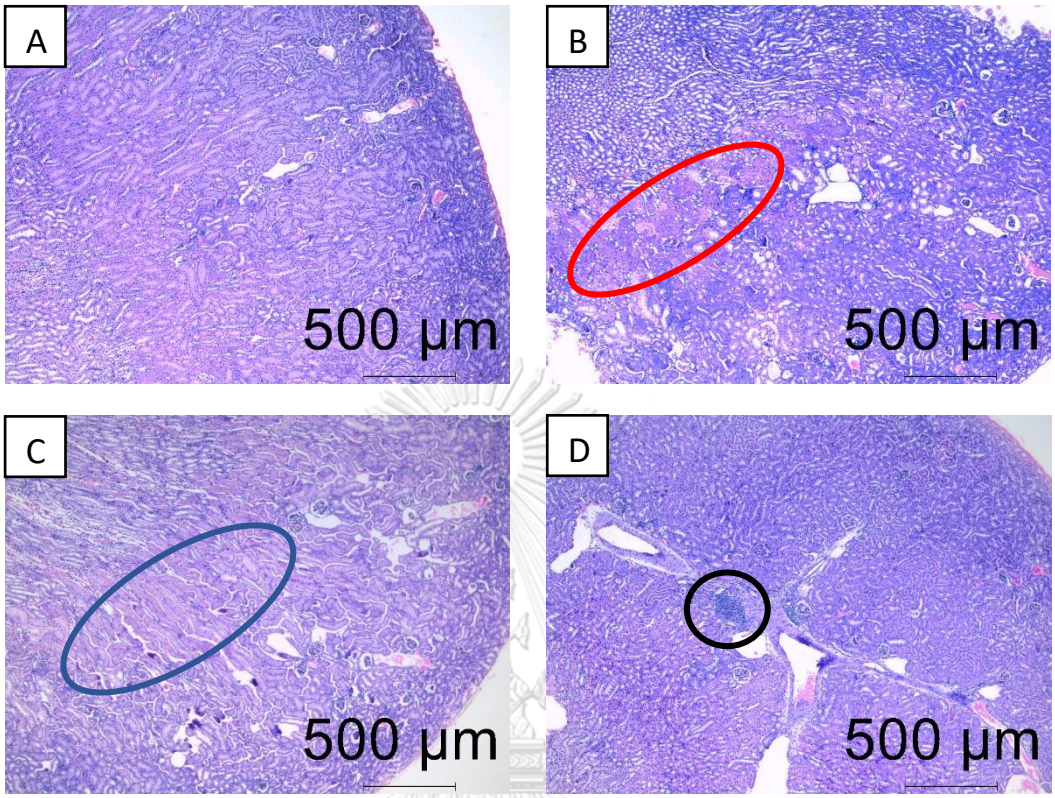

Figure 13. Histopathological changes in kidney sections. The tubular epithelium was minimally (score 0) and mildly hypertrophied (red oval) (score 1) in mice housed in corncob (A) and dried water hyacinth beddings (B), respectively. The moderate tubular hypertrophy (blue oval) (score 2) was found in mouse housed in corncob bedding (C). There was minimally interstitial infiltrates of mononuclear cells (black circle) in the cortex of the kidney (score 1) from mouse housed in aspen shaving bedding (D). Scale bar 500 µm. Hematoxylin and eosin (H&E).

The examples of histopathological findings of liver tissue were presented in Figure 14.

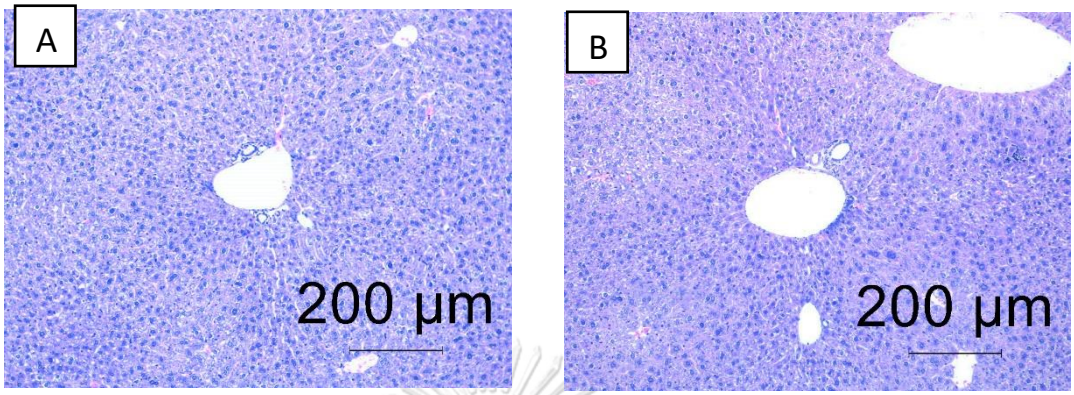

Figure 14. Histopathological findings from liver sections. No remarkable lesion (score 0) was noted in both mice housed in aspen shaving (A) and banana midrib (B) beddings. Scale bar 200 µm. Hematoxylin and eosin (H&E)

Foot pad histopathological finding examples were showed in Figure 15.

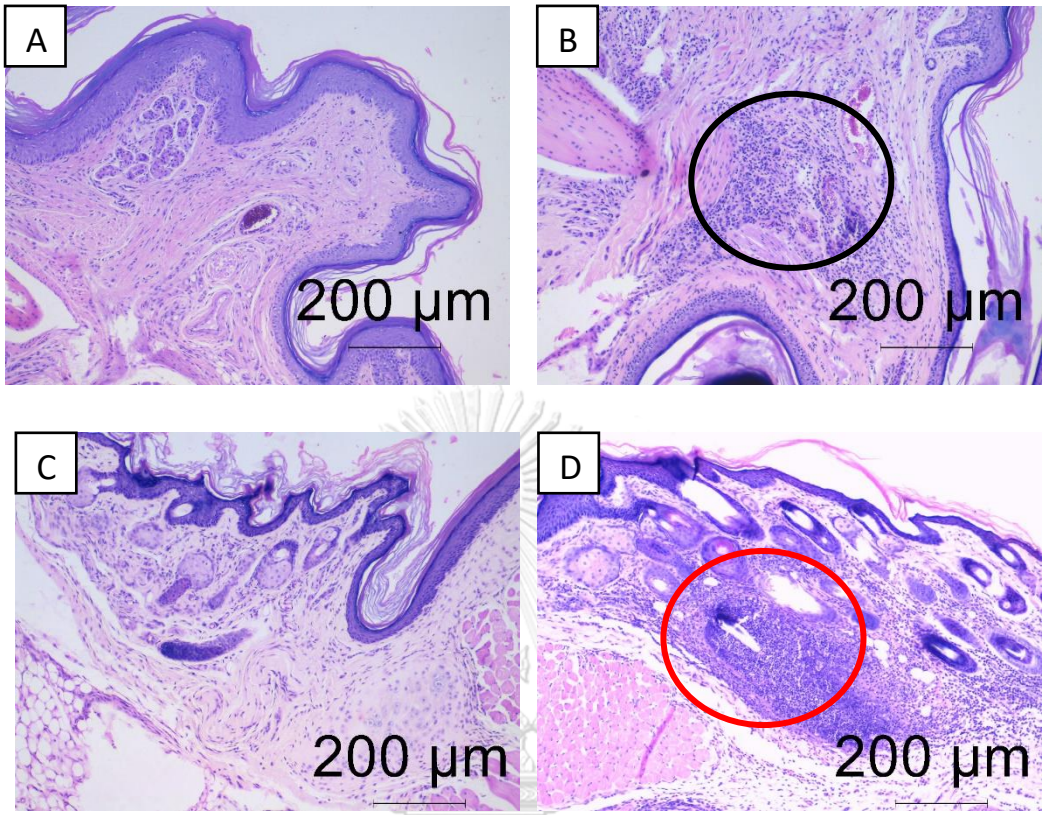

Figure 15. Histopathological changes in foot pad sections. Normal foot pad histopathological sample (score 0) was presented in mouse housed in corncob bedding (A). The superficial dermis of the foot pad that had a focal area of minimal infiltrates of neutrophils (black circle) with a score of 1 was observed in another mouse housed in corncob bedding (B). Normal hair follicle and foot pad epithelium (score 0) was noted in mouse housed in corncorb bedding (C), while focally disrupted of hair follicle with pyogranulomatous inflammation consisting of neutrophils and macrophages (red circle) (score 1) was reported in another mouse from same group (D). Scale bar 200 µm. Hematoxylin and eosin (H&E).

CHAPTER V

DISCUSSION

This study compared the feasibility of using the alternative rodent beddings (dried banana midrib and water hyacinth) with the commonly used rodent beddings (aspen shaving and corncob) housing in the filter top static cage. The study focused on both physical and physiological properties of these beddings.

Part I

Assessment of contamination

Previous study reported that the beddings made from the agricultural by-products were contaminated with several toxins (Lin et al., 2010). Aspen chip contained some sawdust and might be contaminated with the wood preservatives such as penta-chloric phenol, tributylic tin compounds, as well as chromium and copper salts. These contaminants could affect the physiological functions of rodents (Wirth, 1983). Banana midrib could be contaminated with several toxins including heavy metals and pesticides. Corncob had a high level endotoxin causing respiratory syndromes and immunologic responses in rodents (Whiteside et al., 2010). Water hyacinth is usually contaminated with several substances found in that water such as heavy metals (Tiwari et al., 2007) and organic materials (Zimmels et al., 2007). For microorganism contamination, total bacterial count was found to be lowest in the perlite and wood shavings-perlite beddings compared with corncob (Yildirim et al., 2017). Our results showed that there was very low level of contaminations, especially for serious pathogenic organisms, heavy metals, pesticides, and some bioactive substances. Although, arsenic, cadmium and lead were detected in aspen shaving, banana midrib, and dried water hyacinth. These contamination levels were within acceptable levels for animal feed (CODEX, 2010). This low level of contamination might be due to the production process of the vendor and the controlled of using the heavy metal and pesticide in agricultural industry, and the good hygiene of the bedding processing.

Bedding absorbency and surface moisture

In this study, aspen shaving had the highest absorption by volume followed by corncob. Nevertheless, the rank order was not the same as density and absorbency by mass, in which corncob was the densest and the least absorbent by mass. While corncob showed superior surface moisture, compared with other beddings suggesting by the fact that other beddings retained some moisture on some spots for hours of testing. The previous study reported that corncob was the best bedding for absorbency capacity compared with aspen chip, loose pulp bedding, and reclaimed wood pulp (Mason and Burn, 2004), as well as, rice hull (Carbone et al., 2016). However, when corncob was compared with the woodchip and para rubber, corncob had the lowest absorption (Kengkoom et al., 2008). For surface moisture, corncob also had better moisture surface control than rice hull bedding (Carbone et al., 2016). In the present study, banana midrib and dried water hyacinth had lower absorbency by volume, while the surface drying capacity were comparable to aspen shaving and corncob. It could be inferred from this study that banana midrib and dried water hyacinth were relatively hydrophilic. Indeed, the composition analysis of banana midrib and dried water hyacinth indicated that they were high in cellulose and hemicellulose (Liming and Xueliang, 2004; Chen et al., 2008a). Water hyacinth trapped the hydrophilic molecules by capillary action. Dried water hyacinth, made from the stalk of water hyacinth, that has sponge like characteristics which was benefit to the absorbency capacity and mice's foot pad. Due to the variation of bedding density, absorbency by mass was easier to be standardized than by volume. However, the absorbency per unit of volume is the common practice to evaluate the absorption capacity. Moreover, the density and absorptive ability of aspen shaving depend on the sample packing. To control this factor, in this study, a single person gently packed aspen shaving to reduce variation of density in all experiments. In this study, the cage was 11.5 inch length x 7 inch wide x 5 inch height. The costs of bedding per cage were 80.65 baht for aspen shaving, 15.28 baht for banana midrib, 104.02 baht for corncob, and 24.80 baht for dried water hyacinth when used with thickness as in the current study.

The contact surface of the bedding could contribute to tissue injury such as lameness and dermatitis as well as impaired thermoregulation (Wolfensohn and Lloyd, 2008). The fecal contamination with the moisture retain on the bedding surface was a significant risk factor of pododermatitis in rabbits and rodents (Blair, 2013). Therefore, both absorbency and surface moisture are equally important for rodent bedding selection.

Part II

Daily health observation, body weight monitoring, and food and water consumptions

In this study, weekly averaged body weight, body weight gain, as well as food consumptions were not statistically different among groups, while water consumption was statistically different among groups. Also, grimace scales of all mice were scored as zero indicated that all animals showed no sign of pain. Moreover, there was no sign of morbidity and the adhesion of bedding to the skin or eyes of the mice. Similarly, the previous study using corncob, rice hull, recycled wood, and pine shaving showed that each bedding had no effect on animal health during 30-day period of the study (Carbone et al., 2016). Another study also reported that the food and water consumptions were not different among groups of mice housed in the cages with wire mesh, wood ships, shredded filter paper, and sawdust bottoms (Blom et al., 1996). In long termed study, the adult rodents that were kept in the cages containing the common or local beddings did not have significant weight reduction (Burn et al., 2006). For food consumption, another previous mice study reported that food consumption of mice was 3-5 g/mouse/day (Whary et al., 2015). Compared with this study, averaged food consumption of the beddings was 4.53-4.88 g/mouse/day. For water consumption, there was no scientific report about the connection between water hyacinth and water consumption in mice. However, water intake in mice was reported in quite wide range from 3.9 to 8.2 mL/mouse/day, depending on the strains of mice (Bachmanov et al., 2002). Comparing with this range, the water intakes of mice in all groups in this study (i.e., 6.09-8.22 mL/mouse/day) were within the previous report. The statistical significance found in dried water hyacinth group might be individual variation or a physiological result of some compositions in the bedding. Further study on this topic should be performed

to elucidate this finding. Nevertheless, the comparable food and water consumptions found in this study with other mice reports could indicated that the bedding type did not affect food and water intakes in mice.

Also, none of these beddings showed significant effect on daily health observation. This could be a result of very low level of contamination in terms of pesticides, aflatoxins, heavy metals, and pathogenic microbes in our beddings. Moreover, the beddings in this study contain several desirable characteristics of bedding such as good moisture absorbency, inedible appearance, non-traumatic contact, and nontoxic. Besides, the micro and macro environment had been controlled throughout the study. The daily health observation and consumption of food and water are important parts of any laboratory animal study as they can indicate physiological effects of the test substances or treatments. The body weight is also important for medication or test substance calculation, as well as for treatment outcomes evaluation. The 10% reduction of body weight is normally used for considering as the important moribund signs of the study humane endpoint (NRC, 2011).

Intracage ammonia level

The present study demonstrated that intracage ammonia concentrations were significantly different among groups since day 4th after changed bedding. In addition, aspen shaving and banana midrib reached the intracage ammonia level of 25 ppm on day 4th after changed bedding. The intracage ammonia levels of dried water hyacinth were higher than 25 ppm within 3 days. In this study, the intracage ammonia in aspen shaving, banana midrib, and dried water hyacinth groups were sharply increased, which might be due to the low absorbency capacity, the poor surface moisture control, and/or the use of filter top. Moreover, in this study, mice housed in corncob bedding seemed to be less effected by intracage ammonia measured from numbers of affected mice and nasal histopathological grading scores. Therefore, the filter top cage that uses these three types of beddings should be changed the bedding twice a week. While the intracage ammonia levels in corncob were greater than 25 ppm on day 7th after changed bedding. Thus, once a week of bedding changing is enough. According to their bedding changing frequency, the bedding costs per cage for one month in this study were 645.20 baht for aspen

shaving, 122.24 baht for banana midrib, 416.08 baht for corncob, and 198.40 baht for dried water hyacinth. Up to now, the intracage ammonia level for rodents has not been standardized. Previously, the 25 ppm has been used as a guidance (Domer et al., 2012). This data was referred from human safety recommended by the National Institute for Occupational Safety and Health standard (Ferrecchia et al., 2014). However, some publications did not agree with that standard level due to the rodent often live in crowded underground tunnel with limited ventilation (Carter and Lipman, 2018). The factors of increase ammonia accumulation included the high humidity, the type of bedding, and the moisture (Perkins and Lipman, 1995). Although, in this study, room temperature was remained stable at 22±3°C with a relative humidity of 60±10%. The airflow in this study was 10-15 air changes per hour at cage level. However, the filter top used in this study could reduce the flow of air replacement in the cage (Reeb-Whitaker et al., 2001). Therefore, the intracage ammonia level in the IVC should be lower compared with that of the filter top cages with the same type of bedding as the IVC had higher air change per hour. Nevertheless, in this study, all beddings could dry 0.5 mL of the municipal water with green dye on the bedding surface within 4-6 hours, so all of these four bedding types in this study are suitable for mice housing.

Hematology and biochemical analysis

Our result showed that all hematological and biochemical parameters were within normal levels according to the reference ranges from animal facility and previous mice reports (Serfilippi et al., 2003, Traslavina et al., 2010, Watanabe et al., 2014). This could be a result of low contamination of beddings. Also, mice did not ingest these beddings in large volume as there was no significant gastrointestinal impaction from necropsy. Body weight gain was also within the normal range of growth from the animal facility reference. Moreover, food and water consumptions were normal. Nevertheless, the *urate concentrations* of *mice* summarized from 103 studies showed extremely wide variation from 0.002 to 12.8 *mg/dL. This variation could be a result of the anesthesia with ether induced* **α**-vasoconstriction and ischemia leading to degradation of intracellular ATP to uric acid (Watanabe et al., 2014). The hematology profile and biochemistry were very important due to these parameters could reflect the animal health status and any significant changes during subclinical and clinical problems. Mice are common laboratory animals used in

biomedical research and development (Schneck et al., 2000), including pharmacology, safety pharmacology, immunology, toxicology, transgenic, laboratory animal medicine, and genetics. In toxicology studies, analysis of hematology and clinical chemistry parameters is essential (Frith et al., 1980). Therefore, blood analysis study is very important to control the true study outcomes when laboratory plans to change types of beddings or any environment that can affect animal health. However, this study did not evaluate the hematology and blood chemistry at the baseline, thus variation from individual animal could not be excluded.

Histopathological finding: nasal, kidney, liver, and foot pad

Histopathology results of nasal tissue demonstrated that the lesions were mainly seen at the section of 4 mm from the tip of the nose. Main lesions found in this study were neutrophil infiltrations around the affected epithelium, small foci of erosion/ulceration of the respiratory epithelium, focal cytoplasmic vacuole degeneration and hyperplasia of respiratory epithelium, as well as early necrosis of the olfactory epithelium, all of which were presented with various scores in most of the mice housed in aspen shaving, banana midrib, and dried water hyacinth beddings. While in corncob group, only mild neutrophil infiltration and epithelial hyperplasia were detected in two mice. These lesions could be a result of high intracage ammonia levels as the elevated intracage ammonia and carbon dioxide concentrations in cages could affect the respiratory health of the mice (Buckley et al., 1984). From previous study, the acute nasal alterations induced by ammonia inhalation were respiratory epithelial cilia loss, exfoliation or necrosis of epithelium, erosions or ulcers, and submucosal inflammation. Furthermore, chronic ammonia exposure could cause mucosal epithelial hyperplasia, squamous metaplasia, dysplasia, neoplasia, and increased intracytoplasmic granularity, as well as turbinate and septal structure atrophy from cartilage degeneration or necrosis and bone resorption (McInnes and Miller, 2007). Moreover, there are some studies reported the generalized epithelial necrosis, focal congestion, edema, hemorrhage, and inflammatory cell infiltration in nasal tissue of mice housed in recycle woods pulp (Ferrecchia et al., 2014). In agreement with previous studies, this study also found that the inflammation severity of nasal tissue seemed to have the same trend with intracage ammonia level and exposure time. However, the nasal pathology could also come from contamination of endotoxin, dust, and coliform in bedding (Whiteside et al., 2010). Also, increased intracage temperature, humidity, and carbon

dioxide could injure the nasal passage (Perkins and Lipman, 1995). In another rodent study, housing density showed some impact on this pathology (DiVincenti et al., 2012). For example, non-breeding pairs had normal finding to mild alterations. While, breeding pairs and their weanlings had higher nasal lesion scores in terms of turbinate atrophy with erosive, necrotizing and exfoliative rhinitis. Also, breeding trios and their weanlings presented more severity of atrophy and necrotizing rhinitis.

Histopathological changes found in kidney sections were focally lesions in the cortex of the kidney. The examples of the lesions included minimal interstitial infiltration of mononuclear cells found in the kidneys of mice housed in aspen shaving, banana midrib, and corncob, as well as minimal to mild hypertrophy of renal tubules at the outer stripe of the outer medulla of the mice housed in corncob and dried water hyacinth groups. Nevertheless, all of these alterations did not affect renal function clinically as all mice had normal renal panel values from blood analysis. Moreover, there was no data in the literatures about the connection between kidney injury and the use of aspen shavings, banana midrib, corncob, and dried water hyacinth beddings for laboratory animals. However, this study could not confirm that these kidney lesions were related to the bedding types, as these lesions might be a normal finding in mice. From the previous study, the control groups of mice (3 months old) in methoxetamine-treated study showed inflammatory cell infiltration, tubular cell necrosis and glomerular damage (Dargan et al., 2014). On the contrary, another study using male ICR mice 3-8 months old as a control group did not find significant inflammatory cell infiltration in renal histopathological examination in these mice (Yeung et al., 2009). These variations of renal histopathological findings could be a result of several different factors across the studies such as mice strain, age, husbandry, and environment. Therefore, further controlled study with larger animal number and/or longer period of study is required to confirm the effects of beddings on renal histopathological findings.

In this study, no remarkable lesion was seen in the histopathological examination of livers in any group. This finding was correlated with normal health observation and blood analysis in all mice. The very low contaminations of heavy metals and pathogenic microbes in these beddings might be an important factor. Most of corncob and aspen shaving had been used as the beddings for toxicity study. Although, the banana midrib and dried water hyacinth had not been studied in terms of liver injury, this present study indicated that these two types of beddings with low

level of contaminations showed no effect on liver function refered from normal blood biochemistry and histopathology refered from normal liver histopathological finding.

Histopathology of foot pad in this study showed no remarkable lesion of mice's forelimbs and hind limbs in aspen shaving group. On the other hand, the lesions were noted in the banana midrib, corncob, and dried water hyacinth groups. For example, the superficial dermis of the foot pad of mice's hind limbs housed on banana midrib and dried water hyacinth beddings contained a focal area of minimal infiltrates of neutrophils. The hair follicles of mice's forelimb housed in corncob bedding were focally disrupted and surrounded by pyogranulomatous inflammation consisting of neutrophils and macrophages. In previous study, mice housed in either wood shavings or corncob bedding with weekly bedding changing had less skin reactions (e.g., degenerative and inflammatory reactions) than that of twice weekly bedding changing (Yildirim et al., 2017). However, there was no statistically significant difference in foot pad histopathological examination in this study. Therefore, all of the four beddings were safe to be used as rodent beddings in terms of skin irritation and/or comfortable.

Limitation of the study

Firstly, the dust level and intracage carbon dioxide level, as well as sodium component of these beddings were not measured; therefore, the variations found in the blood results, as well as nasal and kidney pathological changes could not be clearly explained. Intracage humidity was also one of the microenvironmental factor that could influence the ammonia level in the cage even the room humidity was controlled. Also, the filter cap used in this study could significantly affect the microenvironment of the cages. Thus, further study related to all of these factors should be performed.

Secondly, the contamination of tetrahydrofurandiols, the linoleic acid derivatives with estrogen properties that could interrupt breeding performance (Markaverich et al., 2002) and neuroendocrine function in rodents (Landeros et al., 2012), were not tested in this study. Therefore, these substances should be tested before the alternative bedding is used as the bedding for breeding colony.

Lastly, this study was conducted for 28 days due to the suitable time for the most toxicological study, so these results might be useful in the acute phase of

laboratory study. For chronic study, further pilot or full extent study are needed to confirm the feasibility of these beddings.

Conclusion

This study provides information on using banana midrib and dried water hyacinth as the alternative beddings for laboratory mice compared with the aspen shaving and corncob. The aspen shaving may be good for bedding since it has the greatest absorbency among 4 beddings, while the corncob may be good for bedding since its surface moisture property seemed to be the best among 4 tested beddings. All of the beddings had no effect on animal health, body weight gain, as well as food consumption. Moreover, hematology and clinical chemistry of all mice were within normal limits. However, the intracage ammonia levels in aspen shaving, banana midrib, and dried water hyacinth groups had risen above the 25 ppm within 3-4 days after beddings were changed. This was also consistent with the results of nasal lesions. Thus, aspen shaving, banana midrib, and dried water hyacinth bedding should be changed twice a week.

Appendix

Daily intracage ammonia concentrations (ppm) in all cage during 28-day study period.

Day	A.1	A.2	B.1	B.2	C.1	C.2	D.1	D.2
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	0.0	0.0	6.7	2.6	0.0	0.0	0.7	24.5
4	34.2	0.0	55.6	91.4	2.1	0.0	5.3	100.0
5	68.0	7.9	94.6	23.8	2.0	1.8	22.2	100.0
6	100.0	14.2	95.0	100.0	11.2	37.3	21.4	84.7
7	44.2	4.2	65.6	81.7	26.2	71.1	27.6	100.0
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	43.8	57.1	35.6	40.0	0.7	0.0	16.4	6.0
11	100.0	88.8	22.1	40.1	8.5	0.0	35.8	20.0
12	100.0	100.0	90.0	80.4	15.1	26.1	54.3	32.7
13	100.0	100.0	88.9	71.5	18.4	100.0	90.2	46.2
14	100.0	100.0	100.0	100.0	100.0	18.9	100.0	45.1
15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17	22.0	26.0	10.7	51.5	9.2	0.0	51.9	30.9
18	40.7	100.0	45.0	60.0	10.7	0.0	100.0	100.0
19	100.0	100.0	55.0	42.0	4.9	0.6	100.0	100.0
20	100.0	100.0	78.8	100.0	24.0	16.0	100.0	100.0
21	100.0	100.0	100.0	100.0	81.4	34.2	100.0	100.0
22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
24	0.0	21.0	2.5	6.5	0.8	0.0	41.7	44.8
25	4.6	81.5	10.8	43.1	6.7	0.0	44.0	25.4
26	15.9	50.0	26.0	59.2	8.7	0.6	34.7	52.0
27	86.9	100.0	50.7	80.0	24.3	14.3	23.4	100.0
28	100.0	100.0	85.0	100.0	55.1	11.1	100.0	100.0

Abbreviations: A = aspen shaving group, B = banana midrib, C = corncob, D = dried water hyacinth

Histopathology grading of 40 male mice (10 mice/group)

Histopathological parameters	Animal No.	Aspen shaving	Banana midrib	Corncob	Dried water hyacinth
Nasal histopathological grading (5 scale)	1	4	2	0	0
	2	3	2	0	3
	3	3	0	1	2
	4	3	1	0	0
	5	3	2	1	2
	6	4	2	0	0
	7	3	3	0	3
	8	3	4	0	2
	9	4	3	0	4
	10	1	1	0	2
Kidney histopathological grading (5 scale)	1	0	1	2	0
	2	1	1	3	2
	3	0	0	2	2
	4	0	0	2	0
	5	1	0	2	1
	6	0	0	0	1
	7	1	1	0	1
	8	0	1	0	0
	9	0	0	0	0
	10	1	1	0	0
Liver histopathological grading (5 scale)	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	9	0	0	0	0
	10	0	0	0	0
Foot pad histopathological grading (5 scale)	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	1
	5	0	0	0	0
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	1	0
	9	0	0	1	0
	10	0	1	0	0

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