1 **Acute and chronic toxicity assessment of benzylpenicillin G residues in** 2 **heat-treated animal food products**

3 **Abstract**

4 The current level of penicillin use and its persisting residues in livestock is potentially
5 concerning; the toxicity of penicillin residues in heat-treated animal food products (HAFP)
6 is yet to be elucidated. In this study, the acute and chronic toxicity of benzylpenicillin G
7 (BPG) residues in HAFP was investigated in a mouse model. The calculated LD₅₀ of BPG
8 heated to cooking temperature (BPHCT) was 933.04 mg kg⁻¹ [b.w.] intraperitoneally
9 corresponding to 3.75 times lower than its prototype. Mice fed on the experimental diet
10 containing heat-treated beef with high BPG levels for 6 months displayed a reduction in
11 body weight and altered serum values indicating for liver and renal function. Further, the
12 organ ratios of intestinal and spleen were increased. Histopathological changes were
13 observed in the liver, lung and parenchyma testis tissue. BPHCT residue induced sperm
14 aberration and micronucleated polychromatic erythrocytes formation. Present results
15 indicate that prolonged exposure of BPHCT at higher levels of residue might have an
16 impact on public health. Importantly the toxic concentrations of BPHCT are relatively
17 high compared with levels that would result from the degradation of antibiotic residues in
18 meat from animals that have received a therapeutic dose of BPG.

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21 **Keywords:** Antibiotic residues; Benzylpenicillin G; High temperature cooking; Acute
22 and chronic toxicity; Long term exposure.

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1. Introduction

The effect of the veterinary drug residues on human health is of increasing concern due to the growing consumption of animal derived products (Baynes et al., 2016). Penicillin, a β -lactam antibiotic, has been widely used in food-producing animals (cattle, pigs and poultry) as a veterinary antibacterial agent to control diseases due to its high bacterial killing efficiency, relatively low toxicity and cost (Edwards and Brownlee, 1946; Dunlop et al., 1998). Penicillins also work as growth promoters to enhance the feed efficiency in animal husbandry (Barton, 2000).

With the development of drug-resistance in bacteria, penicillin is often used at higher dosage rates than those indicated on the manufacture recommendations (Chiesa et al., 2006). The typical dose of penicillin used in cattle by intramuscular injection is approximately 3.5 to 10 times greater than the US approved dose (Payne et al., 2006). It was reported that during the years of 2005-2009, nine countries in the EU consumed a total of 11,342 tonnes antibiotics, of which 18.96% was penicillin, with the majority used in Finland, Netherlands and the United Kingdom (Grave et al., 2012). In 2011, total sales and distribution of penicillin approved for use in food-producing animals was 885 tonnes in the USA (FDA, 2014). In China, 4960 tonnes of penicillin was used in animals during the year of 2013 (Zhang et al., 2015).

With the large amount of penicillin used, there are increased risks of exceeding the Maximum Residue Limit (MRL) range ($4 \mu\text{g kg}^{-1}$ and $50 \mu\text{g kg}^{-1}$ in milk and muscle, respectively (Di Corcia and Nazzari, 2002; Commission, 2009)). According to the USDA National Residue Program report, penicillin residues in excess of legal permitted levels were detected in 22% of the total number of violations in the US (Li et al., 2017a). One study reported that in milk, 28% of collected samples were antibiotic positive, of which 11% were considered non-compliant with current European Union

regulations of penicillin (Junza et al., 2014). Becker et al reported that the highest concentration of penicillin residues in bovine kidney and milk were $1200 \mu\text{g kg}^{-1}$ and $536 \mu\text{g kg}^{-1}$, respectively (Becker et al., 2004). In Italy, Ghidini et al. found that the highest concentration of penicillin residues in milk was $6240 \mu\text{g L}^{-1}$ with the mean concentration of $510.2 \mu\text{g L}^{-1}$ of collected samples (Ghidini et al., 2003). Samanidou et al. reported that the concentrations of penicillin residues in beef were $156 \mu\text{g kg}^{-1}$ and $489 \mu\text{g kg}^{-1}$, which were collected in Greek local markets (Samanidou et al., 2007). Myllyniemi et al. reported that the number of penicillin residues in kidney and muscle samples above $400 \mu\text{g kg}^{-1}$ were 10 and 3, respectively, which were from slaughterhouses around Finland (Myllyniemi et al., 2000). Penicillin residues have been reported in chicken from ‘organic’ farms with a concentration of 1.3 ng g^{-1} , which was labelled as antibiotic-free in Hong Kong local markets (Li et al., 2017b). In some developing countries, the level of penicillin residues in animal derived products may be under reported due to the lack of quality assurance programs (Kabir et al., 2004; Kang’ethe et al., 2005; Babapour et al., 2012). In addition, β -lactams antibiotics were detected in 10% of the urine samples from children aged 8-9 in China, with the highest concentration of 40000 ng mL^{-1} for ampicillin (Wang et al., 2015b).

Penicillin is unstable, with many studies highlighting the heat instability of penicillin in animal derived products. Ludger et al. found that residues from penicillin, penillic, penicilloic and penilloic acids formed in the milk and yoghurt after heat treatment and fermentation by LC-MS/MS detection method (Grunwald and Petz, 2003). Rose et al. suggested that temperature higher than 65°C would affect the structure of penicillin, making its half life varying from 15 to 60 min leading to generation to penicilloic acid as the major breakdown product in cooked food (Rose et al., 1997). Other studies have reported additional adverse effects of penicillin residues in animal derived products. It

has been reported that penicillin residues in food could cause allergic reaction, which is known to be mediated by IgE antibody and the soluble factor like IL-4 or IL-13 (Pene et al., 1988; Dayan, 1993; Punnonen et al., 1993; Pawankar et al., 1997; Guéant-Rodriguez et al., 2006). Mund et al. reported that some severe clinical symptoms such as dermatitis, cutaneous eruptions, anaphylaxis and gastro-intestinal symptoms in humans were the results of penicillin residues in poultry products (Mund et al., 2016). Aarestrup et al. emphasised that penicillin residues could also facilitate the spread of antibiotic resistance genes from animals to humans through food chain (Aarestrup et al., 2008). To our knowledge, there is only one study that reported the toxicity of one major degradation product of BPHCT-benzylpenicilloic acid, which showed toxicity *in vivo* and *in vitro* (Cui et al., 2018).

In this study, we investigated both acute and chronic toxicity studies in mice to understand the possible adverse effect posed by BPHCT to human health.

2. Materials and Methods

2.1 Chemicals and reagents

Benzylpenicillin G (BPG, molecular weight: 356.37; 1.6 million units 0.96 g⁻¹; CAS No.: 61-33-6; purity >99%) was purchased from North China Pharmaceutical Co., Ltd and stored in a dark and dry place at 4 °C for subsequent tests. Phosphate buffered solution (PBS, Cat. No. PYG0021), poly-lysine treated slides (Cat. No. AR1065), mouse interleukin-4 (IL-4, Cat. No. EK0405) and interleukin-13 (IL-13, Cat. No. EK0425) ELISA Kits were purchased from BOSTER Biological Technology Co. Ltd. China. Mouse immunoglobulin E (IgE, Cat. No. 88-50460-22) ELISA kit was purchased from Thermo Fisher Scientific Co. Ltd, USA.

2.2 Preparation of test substance

102 BPG (1.6 million units 0.96 g^{-1}) was dissolved in 2 mL of 0.9% saline solution and
 103 used for the study. Normal diet containing BPHCT and beef were served as the
 104 experimental diets and prepared as follows. Briefly, BPG (480 mg mL^{-1}) was injected
 105 into raw beef ($1 \text{ g kg}^{-1} [\text{bw}] \text{ day}^{-1}$) at different dose levels and boiled with beef for 20
 106 min. The details of dose of BPG and weight of beef used in the study are shown in the
 107 supplementary material. The heat-treated mixtures together with the boiling water were
 108 all added into the normal diet, mixed evenly and reshaped to form the experimental dry
 109 diets. Normal diet mixed with heat-treated beef and 0.9% saline solution was employed
 110 as the negative control.

111 **2.3 Animals**

112 ICR mice were obtained from Laboratory Animal Centre of Jilin University. The
 113 animals were 23-25 g in weight and clinically examined to rule out any ailment and
 114 only ones without any signs of diseases were employed in the further study. Mice were
 115 housed at $22 \pm 1^\circ\text{C}$, 50%-70% humidity and artificial lighting was on 12 h day and night
 116 cycle. Mice were fed with the normal diet and unlimited water. An adaptation period of
 117 at least 7 days was employed before the start of actual dosing. The animal experiments
 118 studies were approved by the Animal Care and Use Committee of Changchun
 119 Experimental Animal Centre, Jilin University. The experiments were designed and
 120 conducted in compliance with the OECD Principles and Good Laboratory Practice
 121 (GLP).

122 **2.4 Analysis of the BPHCT**

123 UPLC-MS/MS method was employed to analyse the degradation products of
 124 BPHCT. The details of the method are shown in the supplementary material.

125 **2.5 Acute toxicity study in mice**

The acute intraperitoneal toxicity study for calculating LD₅₀ was carried out according to the OECD423 guideline (OECD(2002)) and Kaber's method (Wang et al., 2010) with modification. The doses of BPHCT used in oral and intraperitoneal acute tests were calculated according to the original concentration of BPG solution (480 mg mL⁻¹). Mice were administered by gavage with the doses of 5 mg kg⁻¹, 50 mg kg⁻¹, 500 mg kg⁻¹, 1000 mg kg⁻¹, 1500 mg kg⁻¹, 2000 mg kg⁻¹ and 5000 mg kg⁻¹ or injected intraperitoneally with a dose level of 648.00 mg kg⁻¹, 777.60 mg kg⁻¹, 933.12 mg kg⁻¹, 1119.74 mg kg⁻¹ and 1343.69 mg kg⁻¹ of BPHCT. The negative control groups were given a 0.9% normal saline solution. After 14 days observation, mortality caused by oral administration or intraperitoneal injection was calculated. The surviving mice were sacrificed for organ necropsy. To obtain the LD₅₀ value and 95% confidence intervals, the following formula was used according to the Kaber's method with modifications (Wang et al., 2010).

$$LD_{50} = \log^{-1}[x_m - i(\Sigma p - 0.5)]$$

$$X = \log^{-1}(\log LD_{50} \pm 1.96 \times i \sqrt{\frac{\Sigma p(1-p)}{n}})$$

x_m: dosage logarithm for the maximal dosage; i: the difference of dosage logarithm between two nearest groups; p: death rate of mice in each group; X: 95% confidence intervals; n: the number of the mice in each group.

2.6 Six months chronic toxicity study

2.6.1 Experimental design

A total of 80 mice were randomly allocated into 4 groups (10 females and 10 males per group) and five mice were housed in each cage under the same conditions as

described above. All mice were fed with the experimental diets every day. The weight of experimental diets provided was calculated based on the mice body weight. If the experimental diets were completely consumed before the end of a day, the normal diet would be supplied without restriction for the rest of the day. During the 6 months test period, body weight of mice was measured every 3 days in the first month and weekly during the rest of the study period.

2.6.2 *Clinical observation and mortality*

Mice were observed twice a day for changes in fur, eyes, behaviour patterns (e.g., changes in gait or posture), morbidity and mortality, during the course of the experiment.

2.6.3 *Serum biochemical parameters analysis*

Blood from the mice was collected in plain tubes peacefully after overnight fasting. The obtained blood was centrifuged at 3500 rpm for 10 min at 4°C and the serum was carefully transferred to a new tube and stored at -20°C for the subsequent analysis. The serum was analysed by BECKMAN COULTURE UniCelDxC 800 synchron clinical system. The clinical biochemical parameters of mice were determined including blood urea nitrogen (BUN), creatinine (CREA), total cholesterol (TC), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), total protein (TP), albumin (ALB), globulin (GLO), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose (GLU), cholinesterase (CHE), sodium (NA) and potassium (K). The contents of IgE, IL-4 and IL-13 in serum were measured by the corresponding ELISA kits.

2.6.4 *Histopathology*

171 The mice were dissected to necropsy check. The organs including brain, heart, lungs,
172 liver, spleen, kidneys, intestine and testis were carefully collected and examined
173 macroscopically for gross lesions. The epididymides from male and femurs from female
174 mice were also collected for the subsequent experiments. All the organs were weighted
175 in order to calculate the ratios of organ-to-body weight and then the organs from each
176 mouse were kept in 10% formalin for hematoxylin and eosin (HE) staining. Histological
177 changes were examined under a light microscope.

178 *2.6.5 Sperm aberration assay*

179 In this assay, the epididymides from male mice were cut into pieces in PBS (pH 7.2)
180 and smears were prepared according to the method described previously (Wyrobek and
181 Bruce, 1975). The abnormal sperms were scored and the aberration ratios were
182 calculated.

183 *2.6.6 Polychromatic erythrocyte micronucleus formation assay*

184 The femurs from female mice were prepared according to the method described
185 previously (Tang et al., 2012). The polychromatic erythrocytes on the slides were
186 observed using a light microscope. The micronucleus rates in each group were
187 calculated.

188 **2.7 Statistical analysis**

189 Data was analysed with SPSS 20.0 program and the difference between the treatment
190 groups and the control groups was compared. Homogeneity of variances was calculated
191 using Levene's test. If the variance was homogeneous, a one-way analysis of variance
192 (ANOVA) was used to analyse the data. Otherwise, if the variance was not homogenous
193 ($p \leq 0.05$), the identification of the statistical significance of the individual groups was
194 tested by Dunnett's. If the data variable is not normally distributed as required for

195 ANOVA, Kruskal-Wallis test were performed. The statistically significance was
196 considered to be $P < 0.05$. Values were expressed as means $\pm$ SD.

197 **3. Results**

198 **3.1 BPHCT analysis**

199 There were five major degradation products of BPHCT, which were
200 benzylpenicillenic acid, N-(phenylacetyl)glycine, isobenzylpenillic acid,
201 benzylpenillic acid and benzylpenicilloic acid. Some under baseline chromatographic
202 peaks were observed, which might be dimer or different combination polymers of
203 generated degradation products of BPHCT. The results are shown in the
204 supplementary material (Supplementary Figure 1, 2 and Supplementary Table 2).

205 **3.2 Acute toxicity study**

206 There was no mortality found in the oral toxicity test. Regarding observational
207 studies, mice exhibited static, huddled up behaviour, and showed asthenia. Interestingly,
208 mice preferred water to food in the first hour and these clinical symptoms would last for
209 a few hours and disappear before the following day, which was correlated in a dose-
210 dependent manner. A dose level of 1000 mg kg^{-1} was established as the no-observed-
211 adverse-effect level (NOAEL), while the lowest-observed-adverse-effect level
212 (LOAEL) was 1500 mg kg^{-1} for the acute oral toxicity. In the acute intraperitoneal
213 toxicity, the symptoms of BPHCT injected mice were similar with those in the acute
214 oral toxicity study, but some mice in the high dose group had also showed dyspnoeic
215 symptoms or shock. The calculated LD_{50} value was $933.04 \text{ mg kg}^{-1}$ [b.w.] and the 95%
216 confidence intervals were $856.72\text{-}1016.15 \text{ mg kg}^{-1}$ (Table 1).

217

218 Table 1. Acute toxicity of BPHCT administered through intraperitoneal injection in
219 mice

Dose level (mg kg ⁻¹)	Number of tested animals	Number of dead animals
648.00	10	0
777.60	10	2
933.12	10	5
1119.74	10	8
1343.69	10	10

220

221 3.3 Chronic toxicity study

222 3.3.1 Effect of chronic BPHCT administration on the morbidity, mortality and body 223 weight

224 Of the cohort, one female mouse and one male mouse were found crawling in circles
225 around the cage continuously in the 60× and 600× dose group after 2 months feeding;
226 this symptom lasted two weeks in the mouse in the 60× dose group, while it occurred in
227 the mouse in the 600× dose group intermittently for the rest of the test. Mice in 600×
228 dose group seemed abnormally active. At the end phase of the study, there was not a
229 single mouse found to be dead due to drug induced toxicity.

230 There was a significant decrease in body weight in the female test mice compared
231 with those in the control group (Figure 1). In the male mice, the decreased body weight
232 was only found in the 600× dose group while increased body weight was found in the
233 6× and 60× dose groups compared with those in the control group.

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236 Figure 1. Mean body weights of mice fed with BPHCT residue in HAFP.

237 The dose level of different groups were: control groups (0.9% saline solution), 6×

238 groups ($92.5 \mu\text{g } 25 \text{ g}^{-1} \text{ day}^{-1}$), 60× dose groups ($925 \mu\text{g } 25 \text{ g}^{-1} \text{ day}^{-1}$) and 600× dose

239 groups ($9.25 \text{ mg } 25 \text{ g}^{-1} \text{ day}^{-1}$) for 6 months. A. Mean body weights of female test mice.

240 B. Mean body weights of male test mice. The data were expressed in mean±SD.

241 3.3.2 Effect of chronic BPHCT administration on the biochemical serum parameters

242 The biochemical parameter profiles from control and treated mice are shown in Table

243 2. The level of IgE, IL-4 and 13 in both genders were raised compared with those in the

244 control groups, but these data did not present significant difference to the data in the

245 control groups (Table 3).

246

247 Table 2. Effect of 6 months oral administration of BPHCT residue in HAFP on the

248 biochemical values of serum in mice

Parameters	Control	6×	60×	600×
<i>Female</i>				
BUN	10.70±4.02	11.96±3.05	12.15±2.19	11.01±2.67
CREA	15.20±2.30	16.90±2.60	14.70±2.41	15.20±2.78
TC	2.38±0.56	2.49±0.71	2.12±0.75	2.71±0.52
TBIL	1.75±0.33	2.27±0.44*	2.47±0.54**	2.53±0.51**
DBIL	0.85±0.29	0.93±0.31	0.98±0.29	0.97±0.40
IBIL	0.90±0.37	1.34±0.26**	1.49±0.45**	1.56±0.28**
TP	56.60±3.27	55.60±2.55	56.30±2.87	57.20±3.58
ALB	28.40±0.97	27.80±1.62	29.30±0.95	29.60±0.97*
GLO	27.80±2.44	27.80±2.04	26.80±1.93	27.60±2.88
ALT	35.60±7.57	35.10±7.58	42.70±7.17*	48.20±6.71**
AST	129.40±41.39	120.30±20.26	127.40±24.86	140.30±32.31
ALP	74.20±19.01	88.00±27.09	116.20±38.29**	96.80±30.51
GLU	5.56±0.52	5.24±1.02	5.22±0.55	5.36±0.72
CHE	10905.10±2195.43	9653.40±2427.30	10433.10±1230.10	9399.20±1956.07
Na	157.85±2.03	156.18±2.39	157.97±2.53	156.91±1.36
K	6.67±0.63	7.44±0.84**	7.27±0.51*	6.70±0.51
<i>Male</i>				
BUN	10.61±3.76	8.88±1.72	10.15±2.39	13.62±3.41*
CREA	18.20±4.89	17.00±2.54	19.80±2.49	21.30±1.83*
TC	3.49±0.72	3.49±0.57	3.78±0.58	3.50±0.57
TBIL	2.97±0.55	3.04±0.50	3.17±0.50	3.06±1.60
DBIL	1.10±0.31	0.90±0.36	1.03±0.24	1.17±0.54
IBIL	1.87±0.46	2.14±0.33	2.14±0.44	1.99±0.58
TP	52.90±4.04	55.70±5.85	56.20±4.34	54.20±4.49
ALB	24.90±0.99	26.60±1.51**	27.10±1.10**	26.40±0.97**
GLO	27.80±2.53	29.10±2.51	29.30±1.64	28.10±1.20
ALT	67.00±24.72	44.20±5.90**	39.10±9.26**	42.20±8.97**
AST	184.60±37.13	139.20±28.51**	121.10±19.23**	136.20±32.91**

ALP	75.20±27.78	87.00±31.02	64.90±18.55	69.00±20.59
GLU	4.55±1.27	6.53±1.26**	4.52±1.54	4.90±1.75
CHE	4245.50±1312.27	4903.40±1643.02	5539.00±1821.32	4676.30±1819.22
Na	160.03±2.42	158.49±2.93	158.98±3.02	159.49±2.10
K	6.76±0.77	7.44±0.63*	7.55±0.74*	7.25±0.78

The BPHCT with beef were offered daily to mice (n=10 per group) by feeding orally at the dose level: 6× groups, 60× groups and 600× groups, normal diets with cooked beef and 0.9% saline solution serve as the control groups. The data were expressed in mean±SD.

*Significantly different from control group at P < 0.05. **Significantly different from control group at P < 0.01.

BUN=blood urea nitrogen (mmol L⁻¹), CREA=creatinine (umol L⁻¹), TC=total cholesterol (mmol L⁻¹), TBIL= total bilirubin (μmol L⁻¹), DBIL= direct bilirubin (μmol L⁻¹), IBIL=indirect bilirubin (μmol L⁻¹), TP=total protein (g L⁻¹), ALB=albumin (g L⁻¹) GLO=globulin (g L⁻¹), ALT=alanine aminotransferase (U L⁻¹), AST=aspartate aminotransferase (U L⁻¹), ALP=alkaline phosphatase (U L⁻¹), GLU=glucose (mmol L⁻¹), CHE=cholinesterase (U L⁻¹), NA=sodium (mmol L⁻¹), K=potassium (mmol L⁻¹).

Table 3. Effect of 6 months oral administration of BPHCT residue in HAFP on the contents of Immunoglobulin E and Interleukins in the serum of mice

Dose level			Control	6×	60×	600×
Parameters	IgE (μg ml ⁻¹)	Male	4.50±2.05	3.82±3.99	5.43±4.81	5.36±3.21
		Female	5.24±6.33	4.78±5.59	4.51±2.03	6.17±5.30
	IL-4 (pg ml ⁻¹)	Male	22.56±4.55	23.60±11.37	26.63±5.11	25.93±5.53
		Female	17.85±1.49	18.93±2.82	18.63±0.87	19.16±2.37
	IL-13 (pg ml ⁻¹)	Male	20.54±1.47	19.66±0.53	21.02±1.73	28.60±13.04
		Female	21.32±3.34	26.77±12.32	25.43±9.04	25.09±13.22

Data were expressed in mean±SD, n=7.

*Significantly different from control group at P < 0.05. **Significantly different from control group at P < 0.01.

3.3.3 Effect of chronic BPHCT administration on Organ ratios and Histopathology

The data of organ ratios from control and treated mice are shown in the Table 4. The brain ratios in male and female test mice were increased but were not statistically different.

Table 4. Effect of 6 months oral administration of BPHCT residue in HAFP on the organ to body weight ratio in mice

Dose level			Control	6×	60×	600×
Brain	Male		11.00±1.13	10.39±2.14	11.32±1.42	11.36±1.69
	Female		13.01±1.27	14.61±1.42	14.56±2.83	14.96±1.09
Heart	Male		7.16±2.11	6.72±1.06	6.95±1.14	6.27±1.00
	Female		6.51±1.69	6.58±1.25	6.14±1.18	6.82±1.14
Liver	Male		45.64±5.81	40.69±7.87	38.85±2.67**	41.36±4.56
	Female		44.76±11.10	46.49±4.73	41.44±4.60	45.63±3.83
Spleen	Male		2.98±0.52	2.52±0.60	2.61±0.76	3.29±1.18
	Female		3.07±0.79	3.58±0.54	3.61±0.44*	3.91±0.49**
Lungs	Male		7.15±1.11	6.99±0.48	6.44±1.06	6.66±0.88
	Female		7.24±1.13	8.00±1.05	7.18±1.14	7.31±0.72
Kidneys	Male		16.46±2.27	16.26±1.64	15.79±2.02	16.05±2.25
	Female		12.34±1.85	13.58±1.37	13.75±1.74	13.28±1.38
Intestine	Male		76.48±7.56	76.76±5.86	80.66±6.61	87.09±16.25*
	Female		101.69±11.36	100.49±12.97	106.35±16.38	118.02±10.36**
Testes	Male		7.11±1.19	7.14±1.35	7.33±2.31	6.66±1.67

Data were showed with means±SD. The organ ratios were calculated using organ weight/body weight × 1000, n=10.

*Significantly different from control group at P< 0.05. **Significantly different from control group at P< 0.01.

In the 600× dose group, diffusely hepatocytes showed eosinophilic granular cytoplasm, individual hepatocyte, necrosis, lymphocytic and histiocytic inflammatory infiltrate in the liver parenchyma (Figure 2). The pulmonary changes consisted of multifocal lymphocytic, histiocytic and plasmacytic inflammatory infiltration in the parenchyma (Figure 3). Furthermore, in the testis, seminiferous tubules were absent or fewer cellular than the control cases, while the interstitium was broadened. There was diffuse severe necrosis of the spermatogenic lineage cells, decreased number of sustentacular cells and spermatids were absent or infrequent (Figure 4). The changes mentioned above were found in 30% percentage of mice in 600× dose group.

281 Histopathological changes were not found in the control and 6× dose groups and few in
282 the 60× dose groups after 6 months treatment.

283

284

285 Figure 2. Histopathological changes in the liver tissue from control group (A) and 600×
286 dose group (B) (× 400).

287 The lymphocytic and histiocytic inflammatory infiltration (long arrow), necrosis
288 hepatocyte (short arrows) and eosinophilic granular cytoplasm (arrow head and the
289 magnified box) were observed in the liver parenchyma in the 600× dose group. No
290 characteristic histopathological changes were observed in the liver of the control mice.

291

Figure 3. Histopathological changes in the pulmonary tissue from control group (A) and 600× dose group (B) ($\times 400$). Multifocal lymphocytic (long arrow), histiocytic and plasmacytic inflammatory infiltration (short arrows) were observed in the parenchyma in the 600× dose group. No characteristic histopathological changes were observed in pulmonary tissue of the control mice.

Figure 4. Histopathological changes in the testis tissue from control group (A), 6× group (B), 60× dose group (C) and 600× dose group (D) ($\times 400$). In the 600× dose groups, seminiferous tubules (long arrows) were loosened and interstitial broadening. The decreased population of sustentacular cells (short arrows), necrotic spermatids (arrow head) and the fewer spermatids remained within the lobules (the circles) were observed. While, these histopathological changes could be observed less in

the 60× dose group. No characteristic histopathological changes were observed in testis of Control and 6× groups.

3.3.4 Effect of chronic BPHCT administration on sperm aberration rate

Several sperm abnormalities including hookless and amorphous were observed with a high frequency in the 60× and 600× dose groups, and at a lower frequency in the 6× dose group administered with BPHCT residue in HAFP orally for 6 months (Figure 5, Table 5).

Figure 5. Selected microphotographs of sperms in male mice after 6 months oral administration of BPHCT residue in HAFP (×1000).

316 Compared with the control (A) and 6× (B) groups, a significant increase in the sperm
317 aberration rate, such as hookless or amorphous acrosome (short arrows), were observed
318 in the 60× (C) and 600× (D) dose groups.

319

320 Table 5. Effect of 6 months oral administration of BPHCT residue in HAFP on
321 aberration rates of sperm in male mice and polychromatic erythrocyte micronucleus
322 formation rates in female mice

Dose level	Frequency of aberration (%)	MNPCE (%)
Control	1.68±0.22	8.69±1.19
6×	2.11±0.65	10.52±1.33
60×	9.35±1.30**	10.23±4.08
600×	19.41±3.28**	15.97±1.13**

323 Data were expressed in mean±SD, 5000 cells/group, n=5, MNPCE= micronucleated polychromatic erythrocytes.

324 *Significantly different from control group at P < 0.05. **Significantly different from control group at P < 0.01.

325

326 3.3.5 Effect of chronic BPHCT administration on Polychromatic erythrocyte 327 micronucleus formation

328 Compared with the control group, no statistically significant increase in the
329 micronucleus formation was observed in mice feed with experimental diets in 6× and
330 60× dose of BPHCT for 6 months (Table 5). However, the rate of micronucleus
331 formation was readily increased in the 600× dose BPHCT group compared with those in
332 the control group (Figure 6).

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334

335 Figure 6. Selected microphotographs of polychromatic erythrocyte micronucleus in
 336 female mice after 6 months oral administration of BPHCT residue in HAFP ($\times 1000$).
 337 A (Control group) presents few polychromatic erythrocyte micronucleus, short arrow
 338 shows the normal polychromatic erythrocytes in the picture. B ($6\times$ group) and C ($60\times$
 339 dose group) present a lower rate of polychromatic erythrocyte micronucleus (short
 340 arrows) formation. A significant increase in the micronucleus formation was observed
 341 in D ($600\times$ dose group). One or two micronucleus could be observed in one cell.

342 4. Discussion

343 Recent studies have showed that animal derived products containing penicillin
 344 residues exceeding the regulatory safety level have caused an increasing health concern
 345 to consumers globally (Baynes et al., 2016). Many studies have demonstrated that the

346 structure of penicillin is unstable at high temperatures (Tian et al., 2017). However, very
347 few studies have reported the toxicity of penicillin heated to cooking temperature, and
348 the safety of penicillin residues in HAFP. Hence, assessment of the toxicity of penicillin
349 residues in HAFP is required and necessary to advise protocols pertaining to public
350 health. In this study, the toxic effects of BPHCT residue were investigated in a mouse
351 model.

352 In the acute toxicity study, the LD₅₀ of BPHCT was 933.04 mg kg⁻¹. A previous study
353 reported that the LD₅₀ of BPG was 3500 mg kg⁻¹ injected intraperitoneally (Hobby,
354 1968). The result suggested that the toxicity of BPHCT was 3.75 times higher than its
355 prototype.

356 It has been reported that a decrease in body weight has been used to demonstrate the
357 adverse effects of chemicals and drugs (Teo et al., 2002). In the chronic toxicity study,
358 we suggest that the decreases in the mean body weights in test mice were the result of
359 the adverse effects caused by BPHCT. However, the reason for the promotion of growth
360 in 60× dose male group was not clear, which should be subject to further investigation.
361 Abnormal activity and crawling in circles are likely to be neurological symptoms, which
362 may lead to the suppression of body weight growth in the test animals. The BPHCT
363 residues in HAFP may possibly be responsible for the decreased growth and abnormal
364 symptoms in the test mice.

365 Haematological analysis is a relevant part of toxicity evaluation for its higher
366 predictive value for human toxicity (Olson et al., 2000). The levels of BUN and CREA
367 in serum are important markers for kidney damage. The level of BUN increases when
368 the blood volume and Glomerular Filtration Rate decrease (Lee et al., 2007). The level
369 of CREA increases as the filtration rate decreases (Wang et al., 2015a). Present results
370 showed that the levels of BUN and CREA in the serum of 600× dose male group but

not the female group were significantly increased compared with those in the control group. High levels of BUN and CREA indicated that BPHCT in HAFP might affect the Glomerular Filtration function and even cause the kidney damage or necrosis to male kidney cells.

The two transaminases AST and ALT are used as biomarkers to evaluate the function of liver and their elevated values in the serum are indicative of liver damage (Tennekoon et al., 1991). Our results showed increased levels of ALT, AST in female test mice compared with those in the control group, indicating liver damage in these animals. Apart from ALT and AST, the other biomarker-TBIL, is also used to evaluate liver function. TBIL is consisted of DBIL and IBIL, and a high bilirubin concentration may indicate the liver dysfunction (Wang et al., 2014). In our study, these serum parameters changed markedly in test mice compared with those in control groups, which indicated that the liver function might be affected in BPHCT treated mice. Taken together, the results suggested that high dose of BPHCT residues might be toxic to liver, affecting liver function.

Histopathological assessment of the liver showed that the hepatocyte granular cytoplasm, necrotic changes and hepatitis were found in liver tissues in mice in the 600× dose cohort. These histopathological findings may have a correlation with changes in the level of ALT, AST and the bilirubin in the serum of 600× dose mice. These evidences may prove the potential hepatotoxicity of BPHCT. According to our UPLC-MS/MS results, BPG broke down to form benzylpenicillenic acid, N-(phenylacetyl)glycine, isobenzylpenillic acid, benzylpenillic acid, benzylpenicilloic acid and polymers that similar to the result reported previously (Depaolis et al., 1977). These degradation products are highly immunogenic agents due to the rupture of β -lactam ring and the penicilloyl group formation, which bind with protein via a covalent bond to

396 form a completed antigen (Levine and Price, 1964; Wal et al., 1975). We theorised that
 397 the inflammatory cell infiltration and consolidation changes in lungs might be the result
 398 of increasing inflammatory cell stimulated by penicillin degradation products binding
 399 proteins. According to our results, parameters of IgE and interleukins in the test groups
 400 increased with different degrees but not significantly. Here, we hypothesise that the
 401 presence of relatively high level of IgE and interleukins in the serum could be specific
 402 for the degradation products of BPHCT. Interestingly, we didn't find obvious
 403 histopathological evidence in the brain to explain the neurological symptoms of test
 404 mice. However, it had been reported that penicillin was neurotoxic (Nicholls, 1980).
 405 The observed neurological signs of test mice should not be ignored and the mechanism
 406 needs further elucidation.

407 We know that naturally occurring mistakes or exogenous factors such as genotoxicity
 408 chemicals and irradiation may cause sperm aberration or spermatogenic dysfunction
 409 during the differentiation (Bruce et al., 1974). In our test, we observed that the sperm
 410 aberration rates in the 60× and 600× feeding groups were significantly increased
 411 compared with those of the control group. The two test groups displayed characteristic
 412 shapes indicating morphological changes, including hookless and amorphous heads.
 413 This suggests that high concentration of BPHCT residue might affect spermatogenic
 414 tissues. The histopathological study of the testis also showed dead spermatogonia cells
 415 and the decreased population of sertoli and sperm cells in spermatogenic tissues, which
 416 are the evidence to support the possible toxicity of BPHCT on spermatogenic tissues in
 417 mice. On the other hand, the micronucleus assay is considered a preferred method for
 418 assessment of genotoxic effect and chromosome damage caused by exposure to ionizing
 419 radiation or carcinogenic chemicals (Chauhan et al., 2000; Fenech, 2000). Multiple
 420 micronuclei may form as the chromosome lagged behind and fail to incorporate into

421 daughter nuclei during the anaphase of mitosis. In the polychromatic erythrocyte
422 micronucleus assay, the formation rate of micronucleus in the bone marrow cells from
423 600× dose group increased significantly compared with that of the control group. Based
424 on our results, we suggest that higher dose of BPHCT residue might interfere a lagging
425 acentric chromosome fragment and be a potential genotoxicity agent. Therefore, our
426 results suggested that sperm aberration and micronucleus formation caused by
427 degradation products of BPHCT in mice indicates possibilities of sperm toxicity and
428 genotoxicity.

429 This work has clearly demonstrated potential toxic effects of BPHCT *in vivo*. We
430 investigated the potential toxicity of HAFP contaminated with penicillin residues, which
431 has not been studied and remained as a blind spot of the public health authorities and
432 consumers. The five major thermal degradation products of BPHCT contain different
433 functional groups such as carboxyl-, hydroxyl-, sulfhydryl- and carbonyl- group etc.,
434 which could explain the observed toxicity in mice. Polymers formed in the process also
435 need to be paid more attention to due to their complex structures. Among these
436 products, only the toxicity of benzylpenicilloic acid has been evaluated *in vivo* and *in*
437 *vitro* (Cui et al., 2018). More studies are needed to address the toxic effects of these
438 degradation products of BPHCT.

439 According to our pilot study, we found that low dose of BPHCT doesn't show toxic
440 effects in mice. The high doses of BPG (60× and 600× dose) were used in the chronic
441 study to evaluate the potential toxicity after heat treatment but not death or severe
442 suffering based on the OECD guidelines, even though the concentration is significantly
443 higher than those observed in the environment. In some countries, especially in
444 developing countries and some rural areas, BPG residues are a major issue due to its
445 illegal use and poor management of withdrawal period (Babapour et al., 2012).

446 Therefore, the possibility of exposing high dose of BPG residues to the public should
447 not be ignored.

448 In another aspect, there are huge differences in the policies of penicillin usage and
449 residues levels worldwide. Present results may be not valuable to those countries that do
450 not allow the use of penicillin in animal husbandry. Nevertheless, this study
451 systematically reported the toxicity of penicillin residues in animal derived products
452 heated to cooking temperature and their potential threats to public health. It also
453 suggested that although rarely reported, the potential harm posed by other antibiotic
454 residues in food should be investigated.

455 **5. Conclusions**

456 In summary, the toxicity of BPG is increased by over 3.75 times as a result of heat-
457 treatment. The BPG has no significant side effect at the 6× dose level in edible animal
458 tissue after heat-treatment. However, over 60× or 600× dose level may lead to various
459 toxicities, particularly the potential hepatotoxicity and pulmonary toxicity, as well as
460 sperm aberration and micronucleus formation after long time exposure. Taking the
461 thermal process into account, this study provides the toxicity evaluation of antibiotics
462 residue in animal derived products, which would inform the legislation on antibiotic
463 usage guidelines. It is necessary for the government to perform a strict supervision and
464 withdrawal period on the use of penicillin and other heat-unstable antibiotics in animal
465 husbandry.

466

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475 Conflict of interest

476 The authors declare that they have no conflicts of interest.

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