Acute and chronic toxicity assessment of benzylpenicillin G residue in cooked meat

Running title: Acute and chronic toxicity assessment of cooked benzylpenicillin G.

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Abstract

The current level of penicillin use and persisting residue in livestock remains concerning high. However, the toxicity of penicillin residue in cooked animal food product (CAFP) is yet elucidated. In this study, the toxicity of benzylpenicillin G (BP) residue in CAFP was investigated to evaluate the acute and chronic toxicity in mice. The calculated LD_{50} of cooked benzylpenicillin G (CBP) was 933.04 mg kg^{-1} [bw] intraperitoneally corresponding to 3.75 times lower than its prototype. Mice fed the experimental diet containing cooked beef with high BP levels for 6 months displayed a reduction in body weights and with altered serum values for liver and renal functions. The organ ratios of intestinal and spleen were increased. Histopathological changes included necrosis in the liver and inflammatory infiltrate in lungs parenchyma. In testis tissue, seminiferous tubules with a broadening interstitium and decreased population of sertoli and spermatids cells were found. CBP residues induced sperm aberration and micronucleated polychromatic erythrocytes formation. While few changes were found in the middle and low dose groups. Present results indicate that prolonged exposure of CBP at higher levels of residue might affect the public health.

Keywords: Antibiotics residues; Benzylpenicillin G; High temperature cook; Acute and chronic toxicity; Long term exposure.

1. Introduction

The consumption of animal derived products is increasing the need to concern about the effects of the veterinary drug residues on human health [3]. Penicillin, one of β-lactam antibiotics, has been widely used in food-producing animals (cattle, pigs and poultry) as veterinary antibacterial agents to prevent diseases due to its characteristics of anti-bacterial efficiency, economical price and non-toxic therapy dose [10, 11]. Penicillins also work as growth promoters to enhance the feed efficiency and growth promotion in animal husbandry [2].

With the development of drug-resistant in bacteria, penicillin used at the extra-label dosage rates to keep effective in farms became commonly [6]. The typically recommended dose of penicillin used in cattle by intramuscular injection are approximately 3.5 to 10 times greater than the US approved dose [29]. It was reported that during the year of 2005-2009, nine countries in the EU consumed a total of 11,342 metric tones antibiotics, of which 18.96% was penicillin, with the majority of uses in Finland, Netherlands and the United Kingdom [16]. In 2011, total sales and distribution of penicillin approved for use in food-producing animals was 885 tons in the USA [13]. In China, 4960 tons of penicillin was used in animals during the year of 2013 [40].

With the huge amount of penicillin used, it would increase the risk of penicillin residue in meat beyond the MRL range (4 μg kg^{-1} and 50 μg kg^{-1} in milk and muscle, respectively [9]). According to the USDA National Residue Program report, penicillin residue in excess of legal permitted levels were detected in 22% of the total number of violations [23]. In the milk, 28% of collected samples were detected to be penicillin residues, of which 11% were not considered with European Union regulations [20]. In addition, Veterinary or Human β-lactams antibiotics were detected in the urine samples at the detection frequencies of 10% in 8-9 ages of pupils in China [36].

Penicillin is instable, with many studies highlighting the heat instability of penicillin in animal products. Ludger found that residues from penicillin, penillic, penicilloic and penilloic acids formed in the milk and yoghurt after heat treatment and yoghurt fermentation by LC-MS/MS detection method [17]. Rose suggested that temperature higher than 65 □ would affect the structure of penicillin to make its half life varying from 15 to 60 min and penicilloic acid was the major product in cooked food [30]. Depaolis et al. identified the products of penicillin residue in meat under both storage and cooking conditions [8]. In another aspect, there are some studies illustrated the adverse effects of penicillin residue in animal derived products. Dayan reported the allergic reaction caused by penicillin residue in food, posing but one important concern to the public [7]. 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 residue in poultry products [25]. Aarestrup emphazised that penicillin residue could also spread the antibiotics resistance genes from animals to humans through food chain [1]. However, scantly amount of literature have demonstrated the toxicity of penicillin residue in CAFP after prolonged exposure.

In this study, we investigate how heating change the toxicity of CBP. Both acute and chronic toxicity study were performed to get the toxicity information of CBP.

2. Materials and Methods

2.1 Chemicals and reagents

Benzylpenicillin G (BP, molecular weight: 356.37; 1.6million unit $0.96g^{-1}$; 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 □ 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

BP (1.6million unit 0.96 g^{-1}) was dissolved in 2 mL 0.9% saline solution and used for the study purpose. Normal diets contained CBP and beef were served as the experimental diets and prepared as followed. Briefly, BP (480 mg mL^{-1}) were injected into raw beef (1 g kg^{-1} [bw] day^{-1}) at the dose of 3.7 mg kg^{-1} day^{-1} (10× group), 37 mg kg^{-1} day^{-1} (100×) and 370 mg kg^{-1} day^{-1} (1000×group) and boiled with beef for 20 min. The dose of BP and weight of beef used in the study were based on dose of ADI [12], the conversion of animal doses to human equivalent doses [14] and Chinese dietary guidelines (2007), respectively. The cooked mixtures were added into the normal diets evenly and reshape to form the experimental dry diets. Normal diet mixed with cooked beef and 0.9% saline solution was employed as the negative control group.

2.3 Animals

ICR mice were obtained from Laboratory Animal Centre of Jilin University. The animals were 20-25 g in weight and clinically examined to rule out any ailment and only ones without any signs of diseases were employed in the further study. Mice were housed at 22 ℃, 50%-70% humidity and the artificial lighting was 12 h day and night cycle. Mice were fed with the normal diets of laboratory and unlimited water. At least 7 days were the adaptation period before the start of actual dosing. The animal experiments studies were approved by the Animal Care and Use Committee of Changchun experimental Animal centre, Jilin University. The experiments were designed and conducted in compliance with the OECD Principles and Good Laboratory

Practice (GLP).

2.4 Acute toxicity study in mice

The acute intraperitoneal toxicity study for calculating LD_{50} was carried out according to the OECD guideline for 423 [27] and Kaber's method [38] with modified, respectively. The doses of CBP used in oral and intraperitoneal acute tests were calculated according to the original concentration of BP 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 CBP. 0.9% normal saline solution served as the negative control groups. After 14 days observation, mortality of oral administration or intraperitoneal injection was calculated. The survival mice were sacrificed for the organ necropsy. To obtain the LD_{50} values and 95% confidence intervals (X), the following formula was used according to the Kaber's method with few modifications [38].

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

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

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.

2.5 6 months chronic toxicity study

2.5.1 Experimental design

A total of 80 mice were randomly divided into 4 groups (10 females and 10 males per group) and housed five in each cage under the same conditions as described above. All mice were fed with the experimental diets everyday. The weights of experimental diets provided were calculated based on the mice body weights. If the experimental diets were completely consumed before the end of a day, the normal diet would be the complement without restriction for rest of the day. During the 6 months test period, body weights of mice were measured every 3 days at the first month and weekly during the rest period.

2.5.1 Clinical observation and mortality

Mice were observed twice a day for changes in fur, eyes, behaviour pattern (e.g., changes in gait or posture), morbidity and mortality, during the treatment of 6 months.

2.5.2 Serum biochemical parameters analysis

Mice blood was collected in tubes peacefully after overnight fasting. The obtained blood was centrifuged at 3500 rpm for 10 min at 4℃ and the serum was carefully moved to a new tube and stored at -20℃ 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.

2.5.3 Histopathology

The mice were dissected to necropsy check. The organs including brain, heart, lungs, liver, spleen, kidneys, intestine and testis were carefully collected and examined macroscopically for gross lesions. The epididymides from male and femurs from female mice were remained for the subsequent experiment. All the organs were weighted in order to calculate the ratios of organ-to-body weight and then the organs from each mouse were kept in 10% formalin for hematoxylin and eosin (HE) staining. Then examined under a light microscopy to quantified histological changes.

2.5.4 Sperm aberration assay

In this assay, the epididymides from male were cut into pieces in PBS (pH 7.2) and prepared the smears according to the method described previously [39]. The abnormal sperms were scored to calculate the aberration ratios.

2.5.5 Polychromatic erythrocyte micronucleus formation assay

The femurs from female mice were prepared referred to the method described previously [31]. The polychromatic erythrocytes on the slides were observed using a light microscope. The micronucleus rates in each group were calculated.

2.6 Statistical analysis

Data were performed with SPSS 20.0 program. Statistical analyses were based on the contrast of the treatment groups and the control groups. Homogeneity of variances was calculated using Levene's test. If the variance was homogeneous, the one-way analysis of variance (ANOVA) was used to analyse the data. Otherwise, the variance was significant ($p \leq 0.05$), the identification of the statistical significance of the individual

groups was tested by Dunnett's. If the data variable is not normally distributed as required for ANOVA, Kruskal-Wallis test were performed. The statistically significance was considered to be $P<0.05$. Values were expressed as means±SD.

3. Results

3.1 Acute toxicity study

There was no subject mortality found in the oral toxicity test. Regarding observational studies, mice exhibited static, huddled up behaviour, and showed asthenia. Interestingly, mice preferred to drink water but not food in the first hour and these clinical symptoms would last for a few hours and disappeared before the next day, which was correlated in a dose-dependent manner. A dose level of 1000 mg/kg was established as the no-observed-adverse-effect level (NOAEL), while the lowest-observed-adverse- effect level (LOAEL) was 1500 mg kg^{-1} for the acute oral toxicity. In the acute intraperitioneal toxicity, the symptoms of CBP injected mice were similar with those in the acute oral toxicity study, but some mice in the high dose group were also showed dyspnoeic symptoms or shock. The calculated LD_{50} value was 933.04 mg kg^{-1} [bw] and the 95% confidence intervals were 856.72-1016.15 mg kg^{-1} (Table 1).

3.2 Chronic toxicity study

3.2.1 Effect of chronic CBP administration on the morbidity, mortality and body weights

Of the cohort, one female mouse and one male mouse were found crawling in a circle around the cage continuously in the 100× and 1000× dose group after 2 months feeding; this symptom lasted two weeks on the mouse in the 100× dose group, while, this symptom was occurred on the mouse in the 1000× dose group intermittently during the

rest of the test. Mice in 1000× dose group seemed abnormally active. At the end phase of study, there was not a single mouse was found to be dead due to drug induced toxicity.

There was a significant decreased body weight in the female test mice compared to those in control groups the test period (Figure 1). In the male mice, the decreased body weight was only found in the 1000× dose group while increased body weights were found in the 10× and 100× dose groups compared to those in the control group.

3.2.2 Effect of chronic CBP administration on the biochemical serum parameters

The biochemical parameter profiles from control and treated mice are shown in the Table 2. The level of IgE, IL-4 and 13 in both genders were raised compared to those in the control groups, but these data did not present significant difference to the data in the control groups (Table 3).

3.2.3 Effect of chronic CBP 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.

In the 1000× 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 infiltrate 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 is 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 1000× dose group. Histopathological changes were not found in the control and 10× dose groups and few in the 100× dose groups after 6 months treatment.

3.2.4 Effect of chronic CBP administration on sperm aberration rate

Several sperm abnormalities including hookless and amorphous etc. were observed with a high frequency in the 100× and 1000× dose groups, and at a lower frequency in the 10× dose group administered with CBP residue in CAFP orally for 6 months (Figure 5, Table 5).

3.2.5 Effect of chronic CBP administration on Polychromatic erythrocyte micronucleus formation

No statistically significant increase in the micronucleus formation feed with experimental diets in 10× and 100× dose of CBP for 6 months was observed, compared to the control group (Table 5). However, the rate of micronucleus formation was readily increased in the 1000× dose CBP group compared to those in the control group (Figure 6).

4. Discussion

Recently studies have showed that the animal derived products containing penicillin residue exceeding the regulatory safety level causes an increasing health concerns to consumes globally [3]. Many studies have demonstrated that the structure of penicillin is unstable at high temperatures [34]. However, very few studies have reported the toxicity of boiled penicillin, and the safety of penicillin residue in CAFP. Hence, assessment of the toxicity of penicillin residue in CAFP is required and necessary to advise protocols pertaining to public health. In this study, the toxic effects of CBP

residues were investigated in mice model.

In the acute toxicity study, the LD_{50} of CBP was 933.04 mg kg^{-1}. A prior study reported that the LD_{50} of BP was 3500 mg kg^{-1} injected intraperitoneally [18]. We suggested that the toxicity of CBP was 3.75 times higher than its prototype.

It has been reported that a decreased in body weight has been used to demonstrate the adverse effects of chemicals and drug [33]. In the chronic toxicity study, we suggest that the decreases in the mean body weight in test mice were the result of the adverse effects caused by CBP. However, the promotion growth in 100× dose male group was not clear, which should be subject to further investigation. Abnormal activity and crawling in circles are likely to be the neurological symptoms. This may lead to the suppression of body weight and retarded growth in the test animals. The CBP residue in experimental CAFP may possibly be responsible for the decreased growth and abnormal symptoms in test mice. These aspects need further investigation.

Hematological analysis is a relevant part of toxicity evaluation as its higher predictive value for human toxicity [28]. The levels of BUN and CREA in serum are two responsible and important markers for kidney damage. The level of BUN increases when the blood volume and Glomerular Filtration Rate decrease [21]. The level of CREA increases as the filtration rate decreases [35]. Present results showed that the levels of BUN and CREA in the serum of 1000× dose group were significantly increased compared to those in the control group in the male group but not in the female group. High levels of BUN and CREA indicated that CBP in CAFP might affect the Glomerular Filtration function and even cause the kidney damage or necrosis to male in kidney cells.

The two transaminases AST and ALT were used as biomarkers to evaluate the function and damage of liver and therefore will be elevated in the serum when damage

occur to the parenchymal liver cells are damaged [32]. The ratio of ALT/AST was used to ascertain the liver function and higher ratio values indicated the liver injury [24]. Our results showed that the changed levels of ALT, AST and the ratio in serum in test groups might indicate the potential liver damage to female mice. However, the levels of ALT and AST in male mice were decreased, which did not suggest liver injury in male mice. In addition, TBIL is consisted of DBIL and IBIL, and a high bilirubin concentration may indicate the liver dysfunction [37]. Furthermore, the level of ALB in serum was described in correlation with liver's function. In our study, these serum parameters changed markedly in test mice compared to those in control groups, which indicated that the liver's function might be affected in CBP treated mice. Taken together, it was suggested that high dose of CBP residue might be toxic to liver, which might affect liver's function and even cause injury in liver.

Histopathological assessment of the liver showed that the hepatocyte granular cytoplasm, necrotic changes and hepatitis were found in liver tissues in mice present in the 1000× dose cohort. These histopathological findings may have a correlation with changes in the level of ALT, AST, ALB and the bilirubin in the serum of 1000× dose mice. These evidences may proof the potential hepatotoxicity of CBP. Previously, it has been established that penicillin could break down to form isomers of benzylpenicilloic acid, isomers of benzylpenilloic acid, benzylpenillic acid and benzylpenicillenic acid from heating treatment [8]. These cleavage products were highly immunogenic agents due to their penicilloyl group, which bind with protein via a covalent bond to form a completed antigen [22]. We theorised that the inflammatory cell infiltration and consolidation changes in lungs might be the result of increasing inflammatory cell stimulated by penicillin cleavage products binding proteins. Interestingly, we didn't find obvious histopathological evidence in the brain to explain the neurological symptoms of

test mice. However, it had been reported that penicillin was neurotoxicity [26]. The observed neurological signs of test mice should not be ignored and the mechanism needs further elucidation.

We know that naturally occurring mistakes or exogenous factors such as the genotoxicity chemicals or irradiation may cause sperm aberration or spermatogenic dysfunction during the differentiation [4]. In our test, we observed that the sperm aberration rates in the 100× and 1000× feeding groups were significantly increased compared to the rate in the control group. The two test groups displayed characteristic shapes indicating morphological changes, including hookless and amorphous heads. This suggests that high concentration of CBP residues might affect spermatogenic tissues. The histopathological study of the testis also showed dead spermatogonia cells and the decreased population of sertoli and sperm cells in spermatogenic tissues, which are the evidence to support the possible toxicity of CBP on spermatogenic tissues in mice. On the other hand, the micronucleus assay is considered a preferred method for assessment of genotoxic effect and chromosome damage caused by exposure to ionizing radiation or carcinogenic chemicals [5, 15]. Multiple micronuclei may form as the chromosome lagged behind and fail to incorporate into daughter nuclei during the anaphase of mitosis. In the polychromatic erythrocyte micronucleus assay, the formation rate of micronucleus in the bone marrow cells from 1000× dose group increased significantly compared to the rate in the control group. Based on our results, we suggest that higher dose of CBP residues might interfere a lagging acentric chromosome fragment and be a potential genotoxicity agent. Hsieh reported the Ames negative results of amoxicillin after heating in the water [19], However, we suggest that the different structure of two beta-lactam antibiotics might result to the different heating cleavage products. Therefore, our results suggested that sperm aberration and

micronucleus formation caused by cleavage products of CBP in mice indicated substantiates possibilities of sperm toxicity and genotoxicity, which might be a potential risk to human health.

This research has clearly demonstrated potential toxic effects of CBP *in vivo*. However, it should be noted that the toxic effects are present at concentrations that are significantly higher than those observed in the environment. With the BP illegal used and bad management of withdraw period, its residues in animal derived products would be serious. Justify further research, the low dose of CBP are more likely to be found in the cooked animal products. We hypothesize that with long-term exposure and accumulation of CBP, the toxicity might pose a potential threat to the public health. Further the toxic affects of levels exhibited in consumed animals on human health for long time periods of time (20, 30 years) cannot be speculated. In another aspect, there are huge differences in the policies of penicillin usage and residue levels in the worldwide. Present results may be not valuable to those countries, which did not allow the use of penicillin in animal husbandry. In that case, the toxicity of other antibiotics residues in CAFP maybe also cause obvious adverse effects and play a harmful way to the public health.

In summary, the toxicity of BP is increased by over 3.75 times after cooking. The CBP has no significant side effects at the 10 times ADI level after cooking as well as the ADI level. Though, over 100 times or 1000 times ADI level may lead to various toxicities, particularly the potential hepatotoxicity and pulmonary toxicity, as well as sperm aberration and micronucleus formation after long time exposure. Our study first takes the thermal process into account to provide the toxicity evaluation of antibiotics residue in animal derived products, which would allow reference to the legislation on antibiotic usage guidelines. Therefore, it is necessary and required for the government

to perform a strict supervision on the use of penicillin and other heat-unstable antibiotics in animal husbandry and strongly advise to follow labelled use of penicillin administration in veterinary practice in order to keep the public health safe.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Tables

Table 1. Acute toxicity of CBP administered through intraperitoneal injection in 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

Table 2. Effect of 6 months oral administration of CBP residues in CAFP on the biochemical values of serum in mice

Parameters	Control	10×	100×	1000×
Female				
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
ALT/AST	0.28±0.05	0.30±0.07	0.34±0.07	0.36±0.08*
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
Male				
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**
ALT/AST	0.39±0.22	0.32±0.06	0.33±0.07	0.33±0.11
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 CBP with beef were offered daily to mice by feeding orally at the dose level (n=10): 10× groups (3.7 mg kg^{-1} day^{-1}), 100× groups (37 mg kg^{-1} day^{-1}) and 1000× groups (370 mg kg^{-1} day^{-1}), Normal diets with cooked beef and 0.9% saline solution serve as the control groups. The data are 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 CBP residues in CAFP on the contents of Immunoglobulin E and Interleukins in the serum of mice

Dose level			**Control**	**10×**	**100×**	**1000×**
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 $P < 0.01$.

Table 4. Effect of 6 months oral administration of CBP residues in diets on mean organ to body weight ratio in mice

Dose level			**Control**	**10×**	**100×**	**1000×**
	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
Organ ratio		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 ratio is 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.

Table 5. Effect of 6 months oral administration of CBP residues in CAFP on aberration rates of sperm in male mice and polychromatic erythrocyte micronucleus formation rates in female mice

Dose level	**Frequency of aberration (%)**	**MNPCE (%)**
Control	1.68±0.22	8.69±1.19
10×	2.11±0.65	10.52±1.33
100×	9.35±1.30**	10.23±4.08
1000×	19.41±3.28**	15.97±1.13**

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

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

Figure Captions

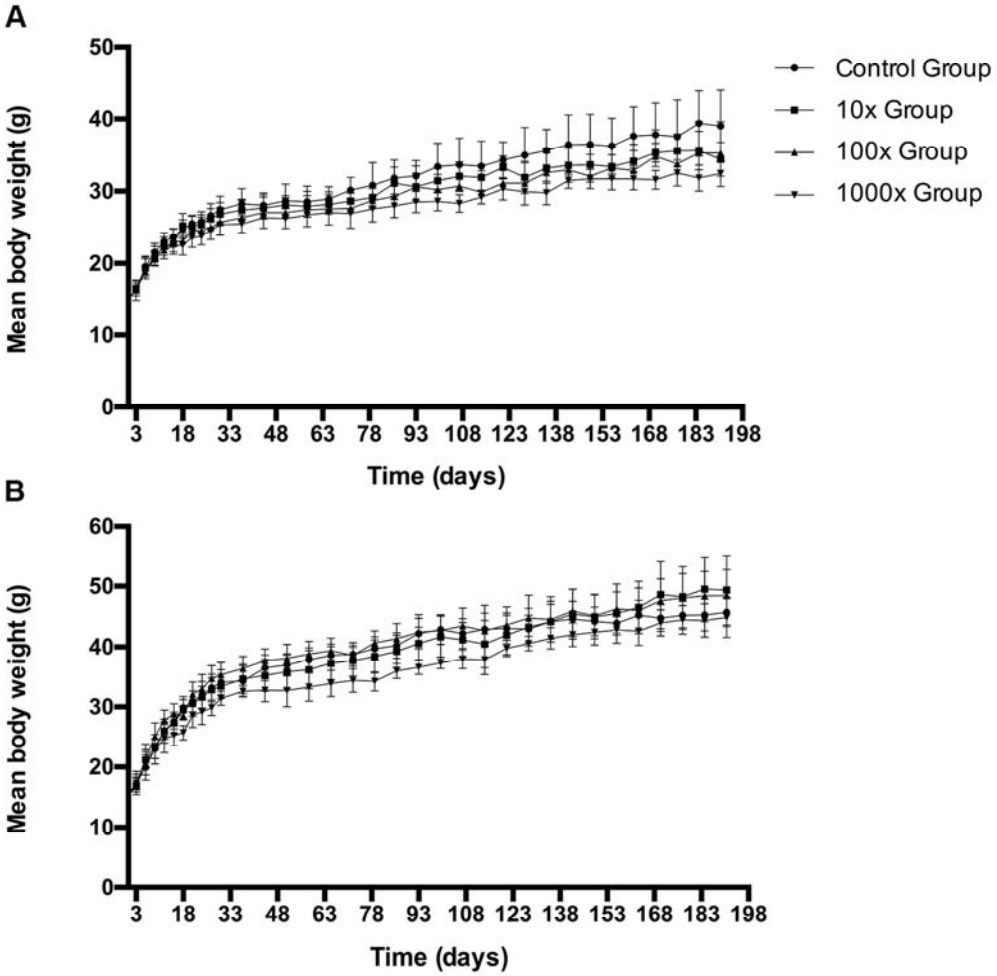

Figure 1. Mean body weights of mice fed with CBP residues in CAFP.

The dose level of different groups were: control groups (0.9% saline solution), 10× groups (3.7mg kg^{-1} day^{-1}), 100× dose groups (37 mg kg^{-1} day^{-1}) and 1000× dose groups (370 mg kg^{-1} day^{-1}) during 6 months. A. Mean body weights of female test mice. B. Mean body weights of male test mice. The data are expressed in mean±SD.

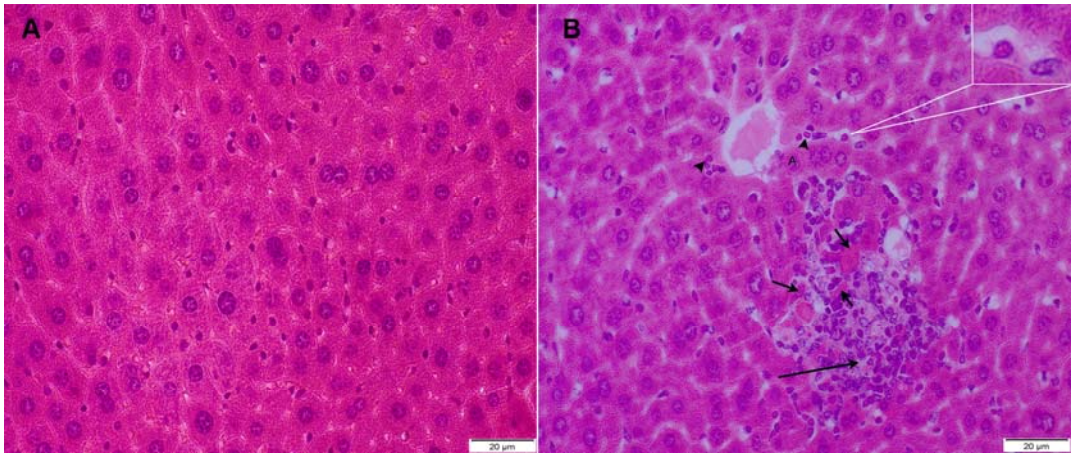

Figure 2. Histopathological changes in the liver tissue (× 400).

The different groups of test animals are shown in (A) control group and (B) 1000× dose group. The lymphocytic and histiocytic inflammatory infiltration (long arrow), necrosis hepatocyte (short arrows) and eosinophilic granular cytoplasm (arrow head and the magnified box) were observed in the liver parenchyma in the 1000× dose group. No characteristic histopathological changes were observed in the liver of control mice.

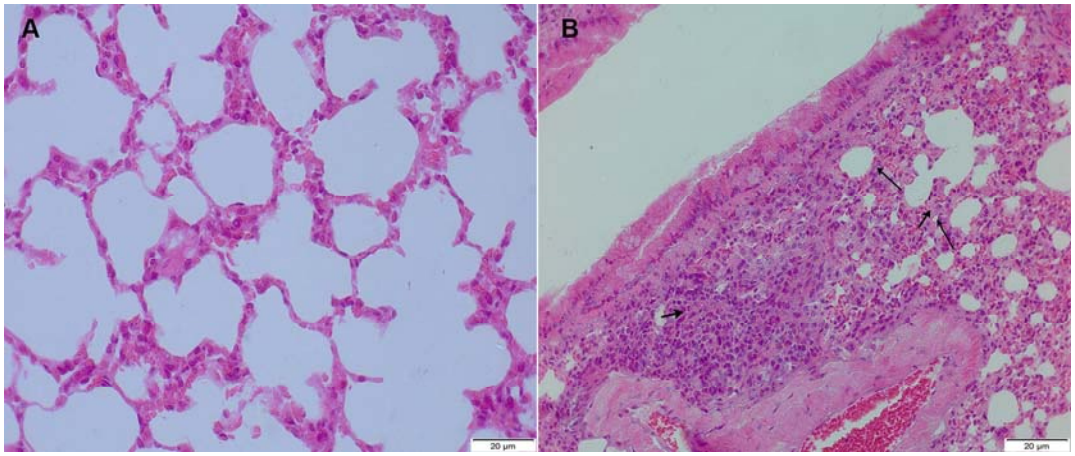

Figure 3. Histopathological changes in the pulmonary tissue (× 400).

The different groups of test animals are shown in (A) control group and (B) 1000× dose group. Multifocal lymphocytic (long arrow), histiocytic and plasmacytic inflammatory infiltration (short arrows) were observed in the parenchyma in the 1000× dose group. No characteristic histopathological changes were observed in pulmonary tissue of control mice.

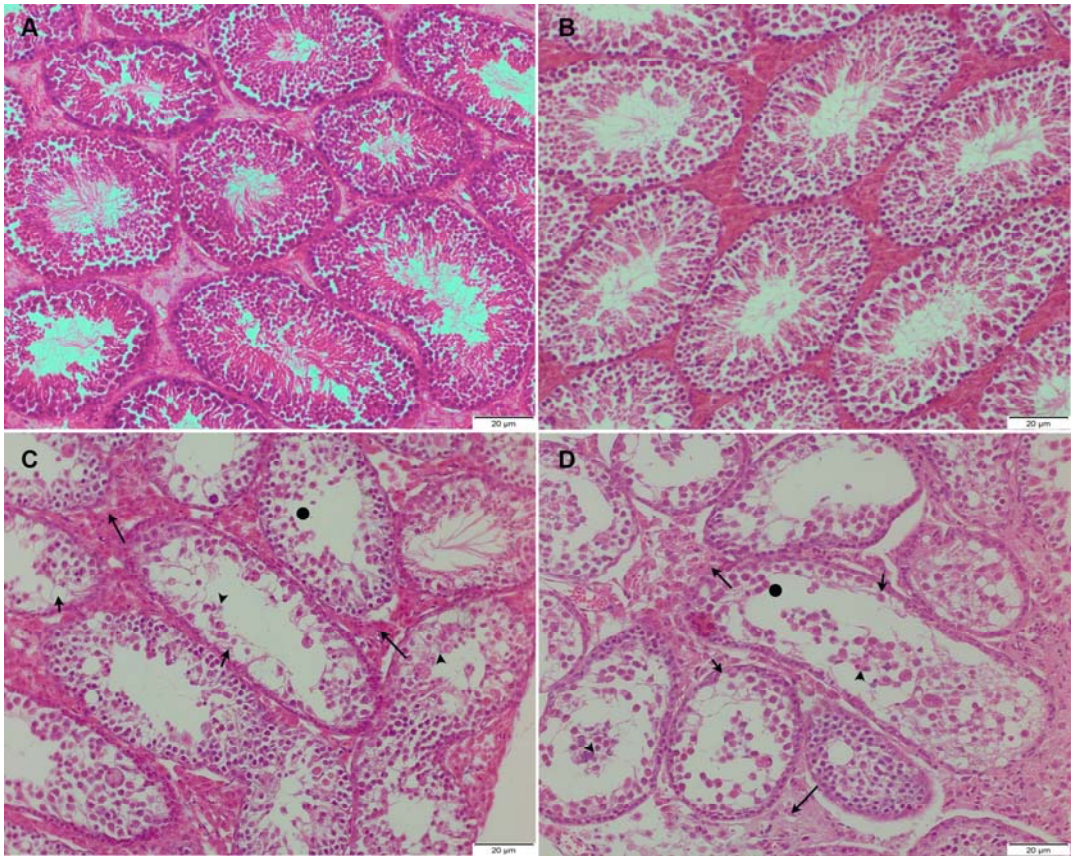

Figure 4. Histopathological changes in the testis tissue (× 400).

The different groups of test animals are shown in (A) control group, (B) 10× group, (C) 100× dose group and (D) 1000× dose group. In the 1000× dose groups, seminiferous tubules (long arrows) were loosed 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 histopathlogical changes could be observed less in the 100× dose group. No characteristic histopathological changes were observed in testis of Control and 10× groups.

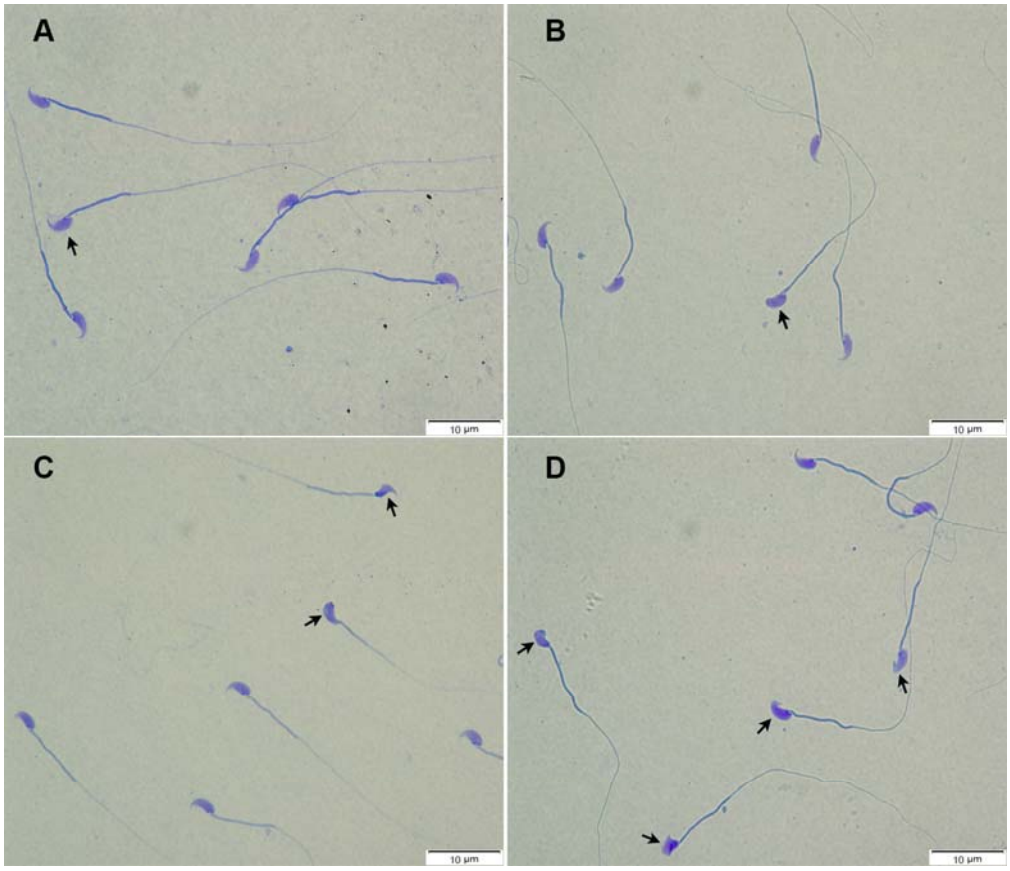

Figure 5. Selected microphotographs of sperms in male mice after 6 months oral administration of CBP residues in CAFP (×1000).

Compared with the control (A) and 10× (B) groups, a significant increase in the sperm aberration rate, such as hookless or amorphous acrosome (short arrows), were observed in the 100× (C) and 1000× (D) dose group.

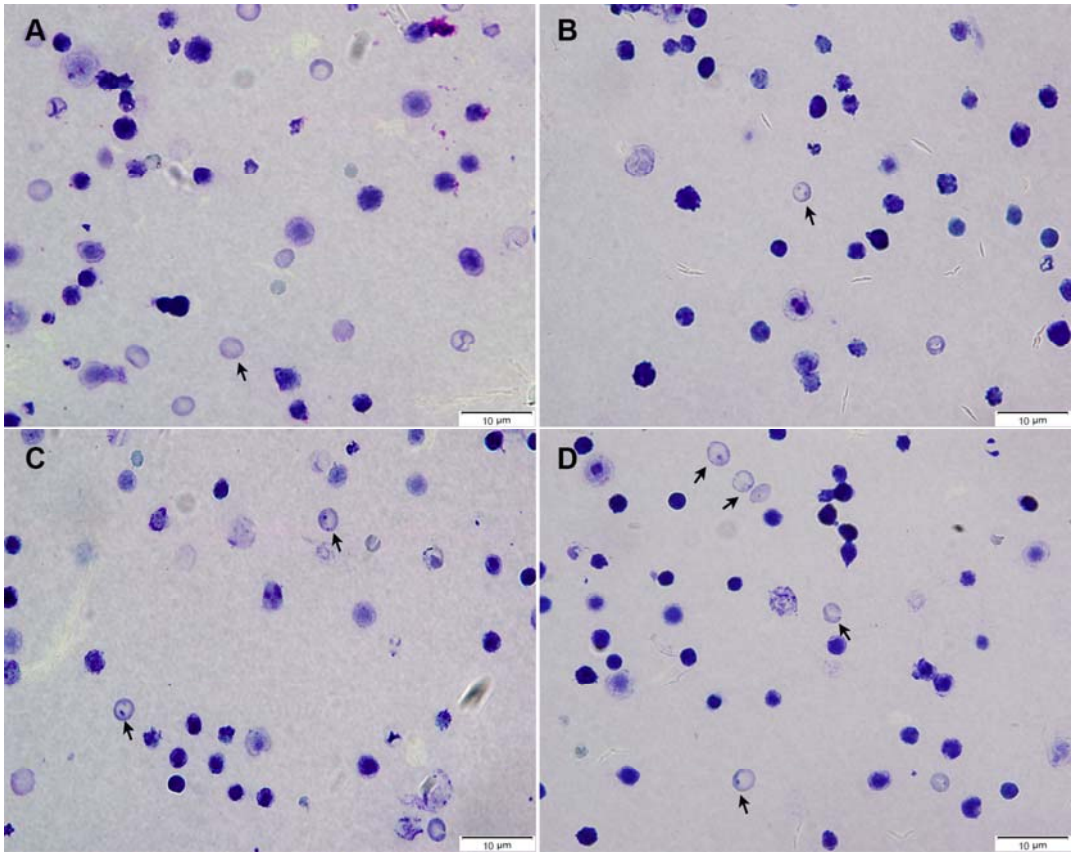

Figure 6. Selected microphotographs of polychromatic erythrocyte micronucleus in female mice after 6 months oral administration of CBP residues in CAFP (×1000). A (Control group) presents few polychromatic erythrocyte micronucleus, short arrow show the normal polychromatic erythrocytes in the picture. B (10× group) and C (100× dose group) present a lower rate of polychromatic erythrocyte micronucleus (short arrows) formation. A significant increase in the micronucleus formation was observed in D (1000× dose group). One or two micronucleus could be observed in one cell.