Preclinical safety of a bionic tiger bone capsule in Sprague–Dawley rats and beagle dogs

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Abstract

Background: Jintiange capsule is composed of bionic tiger bone powder and has similar ingredients to natural tiger bone.

Objective: To characterize the subacute toxicities of Jintiange capsule in rats and beagle dogs for preclinical safety assessment.

Methods: Suspensions of Jintiange capsule were given via gastric lavage over a 26-week period at low (500 mg/kg), mid (1500 mg/kg) and high doses (4000 mg/kg) in SD rats. Beagles were given by gastric lavage of suspensions of Jintiange capsule once daily for 6 days per week for 39 weeks at low (300 mg/kg), mid (900 mg/kg) or high dose (2000 mg/kg).

Results: Repeated gastric lavages of suspensions of Jintiange capsule at doses from 500 to 4000 mg/kg over 26 weeks caused no significant toxicity (No Observed Adverse Effect Level, NOAEL) in rats. In addition, repeated gastric lavages of suspensions of Jintiange capsule at doses from 300 to 2000 mg/kg over 39 weeks caused NOAEL in beagles.

Conclusions: Jintiange capsule was safe in rats at a dose 66.7 times the clinically recommended dose and in beagles at 33.3 times the clinically recommended dose. Our subacute toxicity studies in rats and beagles demonstrated no apparent overall toxicities including haematotoxicities, hepatotoxicities and renal toxicities.

KEYWORDS
beagles, bionic tiger bone capsule, Jintiange, rats, subacute toxicity

1 INTRODUCTION

Osteoporosis is characterised by compromised bone strength and fragility fractures and poses a significant and increasing public health burden with the aging of the population. Despite remarkable progress in our understanding of osteoporosis and development of novel therapeutic agents such as selective oestrogen-receptor modulator (SERM) like raloxifene (Riggs & Hartmann, 2003) and bisphosphonates like alendronate and risedronate (Khosla et al., 2012), the disease still poses a challenge in avoiding long-term safety concerns of currently available drugs, deciding optimal therapeutic regimens and disease management (Tella & Gallagher, 2014; Khosla & Hofbauer, 2017). Traditional Chinese medicine formulas have long been used in the prevention and treatment of osteoporosis (Lin et al., 2017). Chinese medicinal herbs typically exert pharmacological effects through multiple mechanisms targeting multiple pathways, which is consistent with the multiple components of herbal compounds and the presence of numerous biologically active molecules in herbal formulas may exert concerted biological actions without causing significant toxicities (An et al., 2016).
Jintiange capsule is composed of bionic tiger bone powder and has similar ingredients to natural tiger bone (Liu, & Han, 2006). The capsule contains peptides and proteins including collagen and is also rich in calcium and trace elements (Cheng et al., 2003, Takeshi, 1979, Guo et al., 2006). Natural tiger bone has been known to strengthen muscles and bones, expel wind and cold, and relieve pain and used for lumbar pain, knee weakness, lower extremity atrophy and weakness, and difficulty in walking. Bionic tiger bone has been widely used clinically to treat osteoporosis. The interest in and development of bionic tiger bone are partially due to the protected status of tigers (Liu & Han, 2006, Williams, 2015) and studies have demonstrated that bionic tiger bone possesses anti-osteoporotic effects (Wang & Han, 2006). In a rat osteoporosis model, Zhang has shown that Jintiange capsule markedly increases bone mineral density (BMD) of osteoporotic rats and also possesses osteogenic activities by raising plasma calcium, alkaline phosphatase and osteocalcin (Zhang, 2007). Zhao et al. further showed that Jintiange capsule had prominent anti-inflammatory effects in osteoporotic rats (Zhao et al., 2015) and reduced the secretions of osteocalcin and tartrate-resistant acid phosphatase in postmenopausal osteoporotic rats (Zhao et al., 2014).

Though bionic tiger bone has been used clinically for osteoporosis, no subacute toxicity data is available. We carried out the current study to characterise the subacute toxicities of Jintiange capsule in rats and beagle dogs for preclinical safety assessment.

2 | MATERIAL AND METHODS

2.1 | Jintiange suspension

Jintiange power was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China) and dissolved in deionised water to prepare Jintiange suspension at a concentration of 50, 150 and 400 mg/ml for use in rats. The powder was also dissolved in 0.5% sodium carboxymethyl cellulose to prepare Jintiange suspension at a concentration of 60 and 180 mg/ml and in deionised water to prepare Jintiange suspension at a concentration of 400 mg/ml for use in beagles.

2.2 | Animals

One hundred sixty 5- to 6-week-old male or female SD rats, weighing 185.7 to 237.8 g each, and 24 male and 24 female 6- to 8-month-old beagles, weighing 6.9 to 8.1 kg each, were obtained from Charles River Labs, Beijing, China. The animals lived under SPF conditions at an ambient temperature of 20–25°C and 40–70% humidity with a 12 h light-dark cycle and were allowed ad libitum access to water and laboratory chows. The animals were accommodated for 1 week before proceeding to experiments.

The study protocol was approved by the ethics committee of our Center for Drug Safety Evaluation and Research, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal experiments were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the USA NIH.

2.3 | Bodyweight

All animals were individually weighed before administration and weekly thereafter.

2.4 | Food and water consumption

Food and water consumption were measured on the initial day of administration and then weekly. The amounts of food and water were measured before they were supplied to each cage, and the food and water remaining the next day were measured to calculate the difference, which was regarded as daily food and water consumption (g/animal/day).

2.5 | Subchronic toxicity test

The rats were randomly assigned (40 rats/group, 20 rats each sex) to receive deionised H_{2}O (the control group) or Jintiange powder suspension (low dose, 500 mg/kg; mid-dose, 1500 mg/kg; or high dose, 4000 mg/kg) once daily for 26 weeks. The suspension was given by gastric lavage, and the dose-volume was 10 ml/kg. In addition, the beagles were randomly assigned (12 beagles/group, 6 beagles each sex) to receive deionised H_{2}O (the control group), or Jintiange powder suspension (low dose, 300 mg/kg; mid-dose, 900 mg/kg; or high dose, 2000 mg/kg) once daily for 6 days per week for 273 days. The suspension was given by gastric lavage, and the dose volume was 5 ml/kg.

2.6 | Haematological parameters

Blood samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA) and tested at days 92, 183 and 211 using an XT-2000iv Hematology Autoanalyzer (Sysmex, Japan). The following indexes were examined: red blood cells (RBC), haemoglobin (Hb), haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticulocytes, white blood cells (WBC), WBC differential counts (neutrophils, eosinophils, basophils, lymphocytes and monocytes), platelet count, red cell distribution width (RDW), mean platelet volume and mean platelet distribution width. The coagulation tests included prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen using a STAGO Blood Coagulation Analyzer (STAGO, France).

2.7 | Serum biochemistry

The animal blood samples were collected at days 92, 183 and 211 in the tubes containing the coagulator and centrifuged at 500 g for 10 min; the supernatant was then collected into a new tube. The serum
biochemistry indexes included the following: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine kinase (CK), blood urea nitrogen (BUN), creatinine, total protein and albumin. The above parameters were analysed using a Cobas E411 analyser (Roche, Swiss).

2.8 | Urinalysis

An autoanalyser from Roche was used to analyse the urine samples at days 92, 183 and 211. Specific gravity, urine chemistry (pH, glucose, protein, urobilirubin, urobilinogen, ketones, leukocytes and nitrites), occult blood, formed elements and sediments were tested. Sediments were examined under a light microscope.

2.9 | Histopathology

After euthanasia at days 92, 183 or 211, the following organs were isolated from the beagles and rats and enumerated: brain, heart, liver, kidneys, adrenal glands, thymus, spleen, lungs, testis, epididymis, ovaries and uterus. The tissues were fixed in 10% neutral buffered formalin and then dehydrated in gradient alcohol, embedded, sectioned (4µm), and stained with routine haematoxylin-eosin prior to histopathological observations. Histopathological study was made of the following organs of the animals of each group: brain (including the cerebella, cerebellum, and brain stem), spinal cord (cervical, thoracic and lumbar), pituitary gland, adrenal glands, thyroid and parathyroid gland, salivary glands, pancreas, liver, oesophagus, stomach, the small intestine (duodenum, ileum, jejunum), the large intestine (including the colon and cecum), lungs, and trachea, aorta, heart, kidneys, bladder, testis, epididymis, prostate gland, ovaries and fallopian tubes, uterus and cervix, vagina, mammary glands, bone (femur), bone marrow (sternum), skeletal muscle, sciatic nerve, eyes, spleen, lymph nodes (mesenteric lymph nodes) and joints.

2.10 | Ophthalmic examination

In the last week of observation, the external appearance of the eyes of the animals was examined. Mydriasis was induced by dropping a mydriatic into both eyes of each animal. The anterior parts of the eyes, the optic media and the ocular fundus were examined with an ophthalmoscope and a fundus camera for abnormalities.

2.11 | Bone densitometry

After euthanasia at days 92, 183 or 211, the femurs were removed from the animals and examined by densitometry using dual-energy X-ray absorptiometry at Beijing Jishuitan Hospital.

2.12. Statistical analysis

Data were analysed using SPSS 19.0. Shapiro–Wilk test was done to determine whether continuous variables were normally distributed. Normally distributed continuous variables were expressed as mean and standard deviations (SD) while non-normally distributed variables were expressed in median and interquartile range (IQR). Bartlett’s test was undertaken to examine homogeneity of variances among the groups. Data were analysed by one-way analysis of variance (ANOVA) if homogeneity of variances was present among the groups. Otherwise, by Dunnett’s test was done for comparison of multiple groups. Qualitative data were examined by Kruskal–Wallis test and Dunn rank sum test was done for comparison among the groups if Kruskal–Wallis test showed significant difference. p < 0.05 indicated significant statistical difference.

3 | RESULTS

3.1 | General observations

The rats did not show abnormal behaviour during the study period. Mild depilation was observed in several rats. A mobile soft, pliable mass was noticed in the left anterior limb of a rat in the mid-dose group at day 156 and the mass did not enlarge until the day of scheduled euthanasia (Supplementary Table S1). Death was procedure-related and not due to medication. The remaining rats showed no signs of toxicity. Furthermore, there was no statistical difference in BMD among the groups (Supplementary Table S3). In beagles, no statistically significant difference was observed in BMD among the groups. One female beagle in the high-dose group developed muscular fibrillation that persisted for 4 days. No other abnormalities were reported in the beagles.

3.2 | Bodyweight and food intake

The rats showed normal growth in body weight during the study period (Figure 1) and no statistically significant difference was observed in body weight between the control group and the treatment groups as well as among the three treatment groups (p > 0.05). Furthermore, food consumption was largely comparable between the treatment groups and the control group (Supplementary Table S4). Except female beagles in the high-dose group showing significantly higher body weights on several weeks, there was no statistical difference in body weight between the treatment groups and the control group (Figure 1). Food consumption was similar between the treatment groups and the control group.
3.3 | Ophthalmic examination

In the control group and all the treatment groups, there were no gross abnormalities on external eye examinations at days 92, 183 and 221. Ophthalmic examinations using a fundus camera at days 92, 183 and 221 also revealed no abnormalities in the sclera, cornea, iris, lens and the fundus. Furthermore, no abnormalities were observed in ophthalmic examinations of beagles at days 10, 3, 89, 180, 277 and 305.

3.3.1 | Haematology

TT was significantly prolonged in both the mid- and high-dose group of both male and female rats versus the control group at day 92, in the high-dose group of male rats at days 183 and 221 and in the mid-dose group of female rats at day 182 (p < 0.05 or < 0.01) (Table 1). In male rats, at day 221, PT was significantly prolonged in the mid-dose group versus the control group (p < 0.01), aPTT was significantly prolonged in the high-dose group versus the control group (p < 0.05). In female rats, the mid-dose group had a significantly lower mean platelet volume (p < 0.05 vs. the control group) at day 221, and the high-dose group had a significantly lower mean platelet volume (p < 0.01 vs. the control group) and a markedly lower platelet distribution width at day 182 (p < 0.05 vs. the control group). No other coagulation abnormalities including fibrinogen levels and platelet counts were observed in both male and female rats.

No haematological abnormalities were observed in the low-dose group of female rats at days 92, 183 and 211 (Table 1). The haemoglobin concentrations and haematocrit were significantly higher in the mid-dose group and the high-dose group than those of the control group at day 183 (p < 0.01 or 0.05). The high-dose group also had significantly higher RBC counts (p < 0.01) and a higher percentage of lymphocytes than the control group at day 183 (p < 0.05). In addition, the high-dose group had a significantly higher percentage of monocytes at day 211 (p < 0.01). No other haematological abnormalities were reported in the mid-dose group and the high-dose group of female rats.

In male rats, no haematological abnormalities were observed at day 92 in the low-dose group. The percentage of lymphocytes was significantly lower while that of neutrophils was significantly higher in the low-dose group of male rats versus the control rats at day 183 (p < 0.05 or 0.01) and the percentage of reticulocytes was significantly higher in the low-dose group of male rats versus the control rats at day 211 (p < 0.05). In addition, at day 92, in the mid-dose group, RBC counts, haemoglobin concentrations and mean corpuscular haemoglobin concentration were significantly increased compared with those of the control group (p < 0.01). RDW was also significantly increased (p < 0.05 vs. the control group). In the high-dose group, the mean corpuscular haemoglobin concentration was increased (p < 0.01 vs. the control group) while the percentage of reticulocytes was significantly lower than that of the control group (p < 0.05). At day 183, WBC and RBC counts were significantly higher in both the mid- and high-dose group than the control group (p < 0.05 or < 0.01). The RDW was also significantly higher in the mid-dose group (p < 0.05 vs. the control group). In the mid-dose group, the percentage of lymphocytes was significantly lower while that of neutrophils was considerably higher than that of
| Parameters | Control | High dose | High dose |
|------------|---------|-----------|-----------|
|            | Day 92  | D183  | D211       |
|            | Male    | Female  | Male       | Female   |
|            | Thrombin time, s | 29.1 ± 0.9 | 26.7 ± 2.0 | 31.3 ± 1.4* | 31.3 ± 1.7** | 33.0 ± 1.4** | 31.2 ± 0.3** |
| Erythrocytes, 10^{12}/L | 7.53 ± 0.79 | 6.54 ± 2.21 | 8.67 ± 0.47** |
| Haemoglobin, g/L | 135.6 ± 3.6 | 120.8 ± 4.12 | 148.4 ± 6.4** |
| MCHC, g/dl | 18.2 ± 1.7 | 18.5 ± 0.5 | 36.2 ± 0.6** | 36.2 ± 0.4** |
| RDW, % | 17.8 ± 1.0 | 15.5 ± 1.9 | 19.4 ± 1.3* | 18.3 ± 0.6 |
| Reticulocytes, % | 2.96 ± 0.35 | 2.85 ± 0.59 | 2.25 ± 0.46* |
| Leucocytes, 10^9/L | 5.61 ± 1.3 | 2.13 ± 0.94 | 9.37 ± 2.32** | 7.93 ± 1.08** |
| Erythrocytes, 10^{12}/L | 8.57 ± 0.20 | 7.16 ± 0.30 | 9.05 ± 0.39** | 9.00 ± 0.29** | 7.55 ± 0.16** |
| Haemoglobin, g/L | 147.8 ± 3.9 | 132.2 ± 5.4 | 137.5 ± 3.9* | 151.9 ± 5.8 | 138.1 ± 4.1* |
| Haematocrit (%) | 41.2 ± 1.2 | 37.5 ± 1.5 | 40.1 ± 1.7** | 39.6 ± 0.9** |
| Neutrophils (%) | 23.7 ± 5.5 | 21.0 ± 3.3 | 35.1 ± 8.4** |
| Eosinophils (%) | 2.0 ± 0.7 | 2.2 ± 0.5 | 1.2 ± 0.4** |
| RDW (%) | 18.7 ± 0.8 | 13.7 ± 0.6 | 19.7 ± 0.8* |
| PDW (%) | 8.7 ± 0.6 | 8.2 ± 0.4 | 7.8 ± 0.3* |
| MPV (fL) | 7.8 ± 0.4 | 7.7 ± 0.4 | 7.3 ± 0.2** |
| Thrombin time, s | 32.7 ± 2.5 | 30.8 ± 2.3 | 33.1 ± 0.9* | 35.2 ± 1.3* |

Note: Data are expressed as mean ± standard deviation. Only parameters with clinically meaningful changes are shown. Data are not shown for the low-dose group. Only the low-dose group in males had a significantly lower percentage of neutrophils on day 183 (p < 0.01). Abbreviations: APTT, activated partial thromboplastin time; MCHC, mean corpuscular haemoglobin concentration; MPV, mean platelet volume; NE, neutrophils; PDW, platelet distribution width; RDW, red blood cell distribution width.

*p < 0.05.

**p < 0.01 vs. the control group.

The percentage of eosinophils was also significantly lower in the mid-dose group (p < 0.01 vs. the control group).

At day 211, RBC counts and the percentage of lymphocytes were significantly lower in the high-dose group while the percentage of neutrophils was notably higher than that of the control group (p < 0.05 or 0.01). No haematological abnormalities were observed in the low-dose group and the mid-dose group of both male and female beagles at and 89 and the high-dose group of male beagles at day 89 (Table 2). In male beagles, at day 180, the low- and high-dose group had a significantly lower number and percentage of reticulocytes than the control group (p < 0.05). The high-dose group also had significantly prolonged aPTT versus the control group (p < 0.05). In female beagles, at day 89, compared with the control group, the high-dose group had significantly higher RBC, haemoglobin, haematocrit, red cell distribution width and the number and percentage of reticulocytes (p < 0.05 or <0.01) (Table 2). The high-dose group continued to have significantly a greater RBC count and a higher haemoglobin content than the control group at day 180 (p < 0.05).

### 3.4 Serum biochemistry

In male rats, at day 92, in the mid-dose group, the plasma Cl content was significantly higher than that of the control group (p < 0.01) (Tables 3 and 4). In addition, the high-dose group had significantly lower plasma Mg content compared with the control group (p < 0.01). No other clinically meaningful abnormalities in serum biochemistry parameters were observed at day 92.

In male rats, at day 183, the mid-dose group had significantly higher plasma albumin, total bilirubin, and glucose levels versus the control group (p < 0.05 or 0.01). The high-dose group also had significantly higher plasma glucose levels versus the control group (p < 0.01). Both the mid- and high-dose group had significantly lower plasma Na and...
In male rats, at day 211, both the low- and high-dose group had significantly higher plasma Ca levels than the control group ($p < 0.01$). Furthermore, both the low- and high-dose group had notably higher triglyceride levels than the control group ($p < 0.01$). In female rats, all three groups had significantly lower pH in the high-dose group versus the control group ($p < 0.05$). In addition, the high-dose group had markedly increased plasma phosphorus levels ($p < 0.01$). No other abnormalities in serum biochemistry parameters were observed at day 211.

At day 89, the high-dose group of male beagles and the low- and mid-dose groups of female beagles had significantly higher Cl$^-$ than the control group ($p < 0.01$). Furthermore, both the low- and high-dose group had notably higher triglyceride levels than the control group ($p < 0.01$ or $0.05$). At day 180, the plasma osteocalcin level was significantly lower in the mid-dose group than the control group of male beagles ($p < 0.05$). At day 277, the low-dose group of male beagles had markedly higher alkaline phosphatase levels versus the control group ($p < 0.05$) and the mid- and high-dose group at day 277. No other abnormalities were observed.

### 3.5 Urinalysis

Except for significantly lower pH in the high-dose group versus the control group at day 182 in female rats ($p < 0.01$), there were no dose-related changes in the rats’ urinalysis in the subchronic toxicity study.
TABLE 3  Serum biochemistry parameters and bone biomarkers (mean ± standard deviation) of male and female rats receiving repeated gastric lavages of Jintiange powder suspensions

| Parameters          | Control Male | Control Female | Lowdose Male | Lowdose Female | Mid-dose Male | Mid-dose Female | High dose Male | High dose Female |
|---------------------|-------------|---------------|-------------|---------------|--------------|----------------|---------------|-----------------|
| Day 92              |             |               |             |               |              |                |               |                 |
| Cl, mmol/L          | 106.6 ± 1.4 | 1050 ± 1.4    | 108.8 ± 0.9 |              |              |                |               |                 |
| Mg, mmol/L          | 0.886 ± 0.056 | 0.926 ± 0.040 | 0.816 ± 0.019 |              |              |                |               |                 |
| P, mmol/L           | 1.92 ± 0.16 | 1.22 ± 0.19   | 1.63 ± 0.23 |              |              |                |               |                 |
| Day 183             |             |               |             |               |              |                |               |                 |
| Albumin, g/L        | 23.3 ± 0.8  | 32.9 ± 3.4    | 24.6 ± 1.2  |              |              |                |               |                 |
| Total bilirubin, µmol/L | 0.8 ± 0.2 | 1.3 ± 0.4    | 1.3 ± 0.4   | 2.2 ± 1.1    |              |                |               |                 |
| Glucose, mmol/L     | 6.74 ± 0.77 | 6.73 ± 1.18   | 8.87 ± 1.03 |              | 8.68 ± 0.86  | 6.87 ± 0.87    |               |                 |
| Triglyceride, mmol/L | 0.33 ± 0.10 | 0.33 ± 0.08  |              | 0.51 ± 0.16  |              |                |               |                 |
| Na, mmol/L          | 146.9 ± 0.7 | 1434 ± 1.1    | 145.2 ± 1.2 | 1450 ± 1.2   |              |                |               |                 |
| K, mmol/L           | 4.44 ± 0.27 | 4.06 ± 0.26   | 3.89 ± 0.32 | 1067 ± 1.4   |              |                |               |                 |
| Cl, mmol/L          | 108.6 ± 0.8 | 1071 ± 1.7    | 110.3 ± 1.1 |              | 1067 ± 1.4   | 10.7 ± 1.1    |              |                 |
| Mg, mmol/L          | 0.819 ± 0.036 | 0.889 ± 0.070 |              | 0.737 ± 0.056 | 0.795 ± 0.047 |              |               |                 |
| Ca, mmol/L          | 2.16 ± 0.06 | 2.37 ± 0.10   | 2.30 ± 0.05  | 2.29 ± 0.06  |              |                |               |                 |
| P, mmol/L           | 1.63 ± 0.17 | 1.36 ± 0.28   | 2.11 ± 0.25  | 1.89 ± 0.14  |              |                |               |                 |
| N-mid osteocalcin, ng/ml | 17.38 ± 2.85 | 12.30 ± 3.45 |              | 18.75 ± 6.20 |              |                |               |                 |
| D211                |             |               |             |               |              |                |               |                 |
| Aspartate aminotransferase, U/L | 130.6 ± 13.9 | 101.0 ± 17.0 | 97.4 ± 166 |              | 85.6 ± 15.1 |              |               |                 |
| Glucose, mmol/L     | 7.06 ± 0.48 | 7.68 ± 0.60   | 6.87 ± 0.77 |              | 8.31 ± 0.34  |              |               |                 |
| Triglyceride, mmol/L | 0.41 ± 0.16 | 0.68 ± 0.25   | 0.26 ± 0.07 | 0.33 ± 0.12  | 0.27 ± 0.07  |              |               |                 |
| Na, mmol/L          | 146.0 ± 0.8 | 1428 ± 1.0    | 144.9 ± 0.8 | 1442 ± 0.3   | 1480 ± 0.6   | 145.3 ± 0.6   |              |                 |
| K, mmol/L           | 4.35 ± 0.18 | 3.73 ± 0.30   | 3.94 ± 0.19 |              | 14.03 ± 1.4  |              |               |                 |
| Cl, mmol/L          | 107.0 ± 1.1 | 1052 ± 1.1    | 109.9 ± 1.4 |              | 1103 ± 1.4   |              |               |                 |
| Mg, mmol/L          | 0.822 ± 0.041 | 0.878 ± 0.025 |              | 0.958 ± 0.039 |              |              |               |                 |
| Ca, mmol/L          | 2.25 ± 0.06 | 2.41 ± 0.08   | 2.33 ± 0.03 | 2.35 ± 0.04  |              |                |               |                 |
| P, mmol/L           | 1.80 ± 0.08 | 1.19 ± 0.14   |              | 1.72 ± 0.21  |              |                |               |                 |
| N-mid osteocalcin, ng/ml | 21.85 ± 1.82 | 15.13 ± 5.52 |              | 14.08 ± 3.92 |              |                |               |                 |

Note: Data are expressed as mean ± standard deviation. Only parameters with clinically meaningful changes are shown.

*p < 0.05.

**p < 0.01 vs. the control group.
In male rats, at day 210, the heart weight of the high-dose group (1.88 ± 0.13 g) was significantly higher than that of the control group (1.55 ± 0.10 g; p < 0.01). At day 142, the kidney weight of the low-dose group of female rats (2.35 ± 0.13 g) was significantly higher than that of the control group (2.05 ± 0.15 g; p < 0.01). In female beagles, at the end of drug treatment, the thymus weight of the mid-dose group (10.56 ± 2.13 g) was noticeably higher than that of the control group (7.61 ± 1.77 g; p < 0.05). No significant changes were observed in the weights of the other organs in rats or beagles.

In female rats, at day 92, the kidney coefficient was significantly higher in the low-dose group than the control group (p < 0.05 or 0.01) (Supplementary Table S7) while in the mid-dose group, the uterus coefficient was noticeably higher (0.16 ± 0.03 g vs. 0.11 ± 0.03 g; p < 0.05) and the thymus-brain coefficient was also noticeably higher (0.16 ± 0.02 g vs. 0.11 ± 0.03 g; p < 0.05). No other abnormalities were observed in beagles.

3.6 | ECG parameters

There were no dose-related ECG changes in beagles in the subchronic toxicity study.

3.7 | Necropsy

3.7.1 | Organ weights and coefficients

In male rats, at day 210, the heart weight of the high-dose group (1.88 ± 0.13 g) was significantly higher than that of the control group (1.55 ± 0.10 g; p < 0.01). At day 142, the kidney weight of the low-dose group of female rats (2.35 ± 0.13 g) was significantly higher than that of the control group (2.05 ± 0.15 g; p < 0.01). In female beagles, at the end of drug treatment, the thymus weight of the mid-dose group (10.56 ± 2.13 g) was noticeably higher than that of the control group (7.61 ± 1.77 g; p < 0.05). No significant changes were observed in the weights of the other organs in rats or beagles.

In female rats, at day 92, the kidney coefficient was significantly higher in the low-dose group than the control group (p < 0.05) (Supplementary Table S7) while in the mid-dose group, the uterus coefficient was noticeably higher than the control group (p < 0.05). At day 183, in male rats, the lung coefficient was significantly higher in both the mid- and high-dose group than the control group (p < 0.05 or 0.001) (Supplementary Table S8). In female rats, the lung and spleen coefficients were significantly lower in the high-dose group versus the control group (p < 0.05). At day 211, in male rats, the heart coefficient was significantly higher in both the mid- and high-dose group than the control group (p < 0.05 or 0.001). In female rats, the heart coefficient was also markedly higher in the mid-dose group than the control group (p < 0.05).

In male rats, at day 92, the lung-brain coefficient was significantly higher in all three dosing groups compared with the control group (p < 0.05) (Supplementary Table S6). In female rats, the heart-brain coefficient and the spleen-brain coefficient were noticeably lower in the mid-dose group versus the control group (p < 0.05) (Supplementary Table S10). At day 183, the kidney-brain coefficient was significantly higher in the low-dose group compared with the control group (p < 0.01). At day 211, the heart-brain coefficient was significantly higher in the mid-dose group compared with the control group (p < 0.05). No other abnormalities were observed.

In female rats, at day 92, the kidney coefficient of the high-dose group (6.90 ± 0.70 g) was significantly higher than that of the control (8.75 ± 1.00 g; p < 0.05). At the end of drug treatment, the thymus coefficient of the mid-dose group (1.35 ± 0.12 g) was significantly higher than that of the control (0.94 ± 0.18 g; p < 0.05) and the thymus-brain coefficient was also noticeably higher (0.16 ± 0.02 g vs. 0.11 ± 0.03 g; p < 0.05). No other abnormalities were observed in beagles.

3.8 | Histopathological findings

Main histopathological changes of the various organs of rats are shown in Figure 2 and Supplementary Tables S11 and S12. The lungs, spleen, bone marrow, stomach and the intestines, femur, knee joint and ovaries showed normal architecture during the study period. Moderate atrophy was noted in one rat in the mid-dose group at day 211; no other abnormalities were observed. At day 92, the prostate of the control group showed mild infiltration of inflammatory cells (1/5 rat). At day 183, the prostate exhibited mild (2/10 rats) and moderate infiltration of inflammatory cells at in the high-dose group while 3/9 rats in the control group showed normal architecture during the study period. Moderate atrophy was noted in one rat in the mid-dose group at day 211; no other abnormalities were observed. At day 92, the prostate of the control group showed mild infiltration of inflammatory cells (1/5 rat). At day 183, the prostate exhibited mild (2/10 rats) and moderate infiltration of inflammatory cells at in the high-dose group while 3/9 rats in the control group showed normal architecture during the study period. Moderate atrophy was noted in one rat in the mid-dose group at day 211; no other abnormalities were observed.

**TABLE 4** Serum biochemistry parameters and bone biomarkers of male and female beagles receiving repeated gastric lavages of Jintiange powder suspensions

| Parameters                          | Control          | Low dose    | High dose   |
|------------------------------------|-----------------|-------------|-------------|
|                                    | Male            | Female      | Male        | Female      | Male        | Female      |
| Day 89                             |                 |             |             |             |             |             |
| Triglyceride, mmol/L               | 0.44 ± 0.09**   | 0.36 ± 0.05 | 0.54 ± 0.10*| 0.05 ± 0.10*|
| Cl, mmol/L                         | 111.1 ± 4.3     | 112.7 ± 0.5 | 114.5 ± 0.8*| 114.0 ± 0.8*|
|                                   |                 |             |             |             |             |             |
| Day 180                            |                 |             |             |             |             |             |
| N-mid osteocalcin, ng/ml           | 27.40 ± 3.80**  | 22.03 ± 4.29| 18.29 ± 4.20*|
| Day 277                            |                 |             |             |             |             |             |
| Alkaline phosphatase               | 48.7 ± 8.4      | 49.0 ± 14.5 | 96.5 ± 30.0*|

Note: Data are expressed as mean ± standard deviation. Only parameters with clinically meaningful changes are shown.

*p < 0.05 vs. the control group.

**p < 0.01 vs. the control group.

(Supplementary Table S5). In female beagles, the low- and high-dose group had significantly higher urine pH than the control group (p < 0.05 or 0.01) (Supplementary Table S6). No other abnormalities were observed.
FIGURE 2  Representative haematoxylin and eosin-stained histologic sections of various organs in the control rats and rats treated with Jintiange. (a) The heart tissue appears normal structure in both the control rats (left panel) and rats receiving high-dose Jintiange (right panel); (b) the lung tissues appear normal appear normal in both the control rats (left panel) and rats receiving high-dose Jintiange (right panel); (c) the liver tissues are normal in both the control rats (left panel) and rats receiving high-dose Jintiange (right panel); (d) the kidney tissue in the normal control rats (left panel) and in the high-dose group shows mild vacuolation in the renal tubule (right panel); (e) the bone marrow shows normal structure in both the control rats (left panel) and rats receiving high-dose Jintiange; (f) the testis tissue is normal in both the male control rats (left panel) and male rats receiving high-dose Jintiange; (g) the ovaries are normal in both the female control rats (left panel) and female rats receiving high-dose Jintiange. Scale bar, 100 mm.

The pituitary gland of male rats at day 183 (1/9 rat) and 30 (1/5 rat) and in the high-dose group at day 92 (2/5). Ultimobranchial body remnants were found in the control group (1/5 rat) at day 92.

The heart tissues showed mild focal infiltration of inflammatory cells in both the control group and the high-dose group in male rats at days 92, 183 and 221, and the control group at day 183 in female rats. In female rats, the liver exhibited vacuolar degeneration in the control group and the low- and high-dose group at day 92. At day 183, all three treatment groups showed vacuolar degeneration, which was not seen in the control group. At day 211, 1/5 rat in the control group, and 2/5 rats in the mid- and high-dose group exhibited vacuolar degeneration. Microgranuloma was present variably in both the control group and the treatment groups, with no remarkable difference among the groups. Furthermore, necrosis of hepatocytes was present in 1/5 rat in the control group at day 92 and 1/9 and 1/10 rat in the low- and high-dose group at day 183. The epithelia in the renal tubules of female rats showed mild vacuolar degeneration in the control group at days 92, 183 and 221, and in the high-dose group at day 221. Diffuse infiltration of inflammatory cells into the interstitium was variably observed in the control group and the high-dose group of both sexes throughout the study period. Progressive focal nephropathy was seen in 2/10 rats in the high-dose group of male rats at day 183. Protein casts were present 1/5 rat each in the control group and the high-dose group of male rats at day 221. Regenerated epithelial cells of the renal tubules were observed in 5/9 rats in the control group at day 183 and 2/5 rats in the high-dose group of male rats at day 221.

No treatment-emergent histopathological abnormalities were observed in beagles (Figure 3).

4  |  DISCUSSION

Jintiange capsule is composed of bionic tiger bone powder and has been used to treat osteoporosis and other conditions. Though Jintiange capsule has been used clinically for osteoporosis, osteoarthritis and other clinical conditions (Sun et al., 2019, Qi et al., 2017, Wang et al., 2011), preclinical safety data are lacking on Jintiange capsule. The current study demonstrated by repeated administrations of Jintiange capsule via gastric lavage over 182 days in rats and 273 days in beagles that Jintiange capsule caused no apparent treatment-emergent toxicities in SD rats and beagles, indicating that Jintiange capsule was safe preclinically. Jintiange capsule was given at 33 (in beagles) to 66 (in rats) times the clinically recommended doses and caused...
FIGURE 3  Representative haematoxylin and eosin-stained histologic sections of various organs in beagles. (a) The liver tissues in both the control rats (left panel) and rats receiving high-dose Jintiange (right panel) show normal architecture except the presence of microgranuloma. (b) The kidney tissues appear normal with puncta of calcium salt deposits in both the control rats (left panel) and rats receiving high-dose Jintiange (right panel). (c) The ovaries appear normal in the control rats (left panel) and cysts are present in rats receiving high-dose Jintiange (right panel). Scale bar, 100

NOAEL, suggesting that Jintiange capsule is safe for use in humans. Our data provide preclinical guidance on the safe use of Jintiange capsule clinically.

The gastric lavage of suspensions of Jintiange capsule overdoses from 500 to 4000 mg/kg in rats over 182 days and from 300 to 2000 mg/kg in dogs over 273 days were tolerated well, and all planned drug schedules were completed. Six rats died during the study, but none of the death was treatment-emergent. Furthermore, we found no significant difference in food consumption between the control group and the treatment groups in both rats and beagles. No noticeable difference was observed between the control group and the treatment groups in both rats and beagles. We found that the high-dose group of female rats had significantly higher plasma osteocalcin levels versus the control group at day 183 and the high-dose group of male rats had markedly lower osteocalcin levels versus the control group at day 211. In male beagles, at day 180, the plasma osteocalcin level was significantly higher in the mid-dose group than the control group. No noticeable changes in osteocalcin levels were observed at other time points and in other treatment groups or the control group. These findings suggest that Jintiange capsule did not affect osteocalcin in SD rats and beagles. This differed from the study by Zhao et al., who showed that in osteoporotic rats, Jintiange capsule reduced the secretions of osteocalcin (Zhao et al., 2014).

Although we observed noticeable changes in TT and oPPT, RBC counts, haemoglobin concentrations, and mean corpuscular haemoglobin concentration and other haematological parameters between the treatment group and the control group over the course of the study, these changes were not dosed dependent in the treatment groups and did not progress over time, indicating that these haematological changes were of no toxicological significance. Consistently,
no significant dose- and time-dependent changes were observed in beagles. These findings indicate that Jintiange capsule causes no significant haematological toxicities. We also found no significant treatment-emergent changes in ALT and AST and other indicators of liver function in rats. Though histological examination showed variable pathological changes in the liver including vacuolar degeneration, microgranuloma and necrosis of hepatocytes, these changes occurred randomly across both the control group and the treatment groups and showed no dose and time-dependent effects. These observations suggest that Jintiange capsule causes no significant hepatotoxicities. Except for significantly lower pH in the high-dose group versus the control group at day 183 in female rats, no dose-related changes were noticed in the rats’ urinalysis in the subchronic toxicity study. In female beagles, the low- and high-dose group had significantly higher urine pH than the control group. No other abnormalities were observed. Serum biochemistry also revealed no significant changes in renal function indicators like blood urea nitrogen and creatinine. Renal pathology showed mild vacuolar degeneration in both the control group at days 92, 183 and 221, and in the high-dose group at day 221. Diffuse infiltration of inflammatory cells into the interstitium was variably observed in the control group and the high-dose group of both sexes throughout the study period. These renal changes were not dosed and time-dependent and therefore, unlikely treatment-emergent. No significant changes in the renal tissues were observed in beagles. These findings suggest that Jintiange capsule causes no significant renal toxicities. The above findings are consistent with the safety of Jintiange capsule in primary osteoporosis patients (Huang et al., 2014).

Our subacute toxicity studies of in rats and beagles demonstrated no apparent overall toxicities including haematotoxicities, hepatotoxicities and renal toxicities. Our data indicate that Jintiange capsule is safe preclinically and can be further tested in humans.

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CONFLICT OF INTEREST
The authors of this work have nothing to disclose.

AUTHOR CONTRIBUTION
Shu-Fang Wan and Yong-Biao Guan contributed to the study conception and design. All authors collected the data and performed the data analysis. All authors contributed to the interpretation of the data and the completion of figures and tables. All authors contributed to the drafting of the article and final approval of the submitted version.

ETHICS STATEMENT
The study protocol was approved by the ethics committee of the National Beijing Center for Drug Safety Evaluation and Research, The centre is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal experiments were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the USA NIH.

DATA AVAILABILITY STATEMENT
The data sets used or/and analysed during the current study are available from the corresponding author on reasonable request.

PEER REVIEW
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REFERENCES
An, J., Yang, H., Zhang, Q., Liu, C., Zhao, J., Zhang, L., & Chen, B. (2016). Natural products for treatment of osteoporosis: The effects and mechanisms on promoting osteoblast-mediated bone formation. Life Sciences, 147, 46–58.
Cheng, D., Long, P., Zhou, H. T., & Long, Y. S. (2003). Recent research on traditional Chinese medicine in the treatment of osteoporosis. Chinese Journal of Osteoporosis, 9(1), 86–89.
Guo, X. Q., Ye, J., & Li, J. G. (2006). Advances in research on tiger bones and their substitutes. Journal of Shaanxi Normal University (Natural Science Edition), 034(021), 218–221.
Huang, H. L., Xiong, W., & Liu, L. J. (2014). Analysis of the therapeutic effect and the safety of Jintiange capsules in treatment of primary osteoporosis. Chinese Journal of Coal Industry Medicine, 3, 42.
Khosla, S., Bilezikian, J. P., Dempster, D. W., Lewiecki, E. M., Miller, P. D., Neer, R. M., Recker, R. R., Shane, E., Shoback, D., & Potts, J. T. (2012). Benefits and risks of bisphosphonate therapy for osteoporosis. The Journal of Clinical Endocrinology and Metabolism, 97(7), 2272–2282.
Khosla, S., & Hofbauer, L. C. (2017). Osteoporosis treatment: Recent developments and ongoing challenges. The Lancet Diabetes & Endocrinology, 5(11), 898–907.
Lin, J., Zhu, J., Wang, Y., Zhang, N., Gober, H. J., Qiu, X., Li, D., & Wang, L. (2017). Chinese single herbs and active ingredients for postmenopausal osteoporosis: From preclinical evidence to action mechanism. BioScience Trends, 11(5), 496–506.
Liu, Z., & Han, D. W. (2006). Clinical research progress of tiger bone and artificial tiger bone. Chinese Journal of Traditional Medical Traumatology & Orthopedics, 014(002), 73–75.
Qi, Y. J., Cai, J., Guang, L., Shen, X. Q., & Gu, X. M. (2017). Effect of zoledronic acid combined with Jintiange capsule on treatment of postmenopausal osteoporosis. Chinese Journal of Endocrine Surgery, 11(5), 404–408.
Riggs, B. L., & Hartmann, L. C. (2003). Selective estrogen-receptor modulators – Mechanisms of action and application to clinical practice. The New England Journal of Medicine, 348(7), 618–629.
Sun, J., Yang, X. G., & Hu, Y. C. (2019). Efficacy of Jintiange capsules in the treatment of osteoporosis: A network meta-analysis. Orthopaedic Surgery, 11(2), 176–186.
Takeshi, S. (1979). Components and pharmacology of tiger bone. Clinical study of Chinese Medicine, 26(9), 33.
Tella, S. H., & Gallagher, J. C. (2014). Prevention and treatment of postmenopausal osteoporosis. The Journal of Steroid Biochemistry and Molecular Biology, 142, 155–170.
Wang, Q. Y., & Han, D. W. (2006). Advances in the pharmacological effects of artificial tiger bone meal. *The Journal of Traditional Chinese Orthopedics and Traumatology*, 018(011), 70–71.

Wang, J. P., Zhang, J. H., Wang, H. J., Yang, D., & Zhang, S. G. (2011). Analysis on the clinical efficacy of Jintiange capsule for the treatment of osteoarthritis. *Chinese Journal of Hospital Pharmacy*, 31(10), 848–850.

Williams, V. L. (2015). Traditional medicines: Tiger-bone trade could threaten lions. *Nature*, 523(7560), 290.

Zhang, Y. S. (2007). Effects of Jintiange capsule on osteoporosis in rats induced by retinoic acid. *Chinese Remedies & Clinics*, 7(9), 688–689.

Zhao, Y. X., Zhang, B., Zhan, M. S., Wang, W. Y., Yang, J. Q., Hu, Y. F., Haiping, S., & Xuan, W. (2014). Effects of jintiange capsule on the expression of BGP and TRACP in rats with osteoporotic fracture. *Chinese Journal of Osteoporosis*, 20(11), 1302–1305.

Zhao, Y. X., Zhang, B., Zhan, M. S., Wang, W. Y., Yang, J. Q., & Hu, Y. F. (2015). Effects of jintiange capsule on serum il-2, il-4 and TNF-leum expression in rats with osteoporotic fracture. *Chinese Journal of Osteoporosis*, 21(07), 33–37.

**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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