

# Deteriorating Effect on Bone Metabolism and Microstructure by Passive Cigarette Smoking Through Dual Actions on Osteoblast and Osteoclast

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**Abstract** There is no clear evidence to show the direct causal relationship between passive cigarette smoking and osteoporosis. Furthermore, the underlying mechanism is unknown. The objective of this study is to demonstrate the effects of long-term passive cigarette smoking on bone metabolism and microstructure by a mouse model and cell culture systems. BALB/c mice were exposed to 2 or 4 % cigarette smoke for 14 weeks. The bone turnover biochemical markers in urine and serum and also the bone micro-architecture by micro-CT were compared with the control group exposed to normal ambient air. In the cell culture experiments, mouse MC3T3-E1 and RAW264.7 cell lines to be employed as osteoblast and osteoclast, respectively, were treated with the sera obtained from 4 % smoking or control mice. Their actions on cell viability,

differentiation, and function on these bone cells were assessed. The urinary mineral and deoxypyridinoline (DPD) levels, and also the serum alkaline phosphatase activity, were significantly higher in the 4 % smoking group when compared with the control group, indicating an elevated bone metabolism after cigarette smoking. In addition, femoral osteopenic condition was observed in the 4 % smoking group, as shown by the decrease of relative bone volume and trabecular thickness. In isolated cell studies, osteoblast differentiation and bone formation were inhibited while osteoclast differentiation was increased. The current mouse smoking model and the isolated cell studies demonstrate that passive cigarette smoke could induce osteopenia by exerting a direct detrimental effect on bone cells differentiation and further on bone remodeling process.

**Keywords** Cigarette smoking · Osteopenia · Mouse passive smoking model · Osteoblast differentiation · Osteoclast differentiation

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## Abbreviations

|        |   |
|--------|---|
| ALP    | Alkaline phosphatase  |
| ALT    | Alanine transaminase  |
| AST    | Aspartate aminotransferase                                    |
| BMD    | Bone mineral density  |
| BV/TV  | Bone volume per tissue volume                                 |
| Ca     | Calcium   |
| Cr     | Creatinine  |
| DPD    | Deoxypyridinoline   |
| μCT    | Micro-computed tomography                                     |
| MTT    | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| P      | Phosphorus  |
| SMI    | Structural model index  |
| Tb. N. | Trabecular number   |

|         |                                     |
|---------|-------------------------------------|
| Tb. Sp. | Trabecular spacing                  |
| Tb. Th. | Trabecular thickness                |
| TRAP    | Tartrate-resistant acid phosphatase |

## Introduction

The harmful and serious outcomes of cigarette smoking have been implicated in different diseases, such as coronary heart disease [1], lung cancer [2], and chronic obstructive lung disease [3]. Cigarette smoking also causes premature death, and places a huge financial burden on society. Meanwhile, it has recently been shown that cigarette smoking is also a risk factor of osteoporosis, a common disorder in elderly [4]. In the past 20 years, extensive clinical studies were performed to elucidate the association between smoking and osteoporosis. Cigarette smoking is positively correlated with lower bone mineral density (BMD) in both elderly men and women [5], perimenopausal women [6], and even in young men [7]. It is also notable that even secondhand smoke exposure (a form of passive smoking) correlates well with an increase in odds ratio in femoral neck in postmenopausal non-smokers [8]. The impact of secondhand smoking may be even stronger than active smoking, because unlike active smoking, secondhand smoking contained both filtered mainstream smoke and, more importantly, unfiltered sidestream smoke. According to a survey conducted in 2004, it was estimated that in an average, 40 % of children, 33 % of male and 35 % of female non-smokers were exposed to secondhand smoking worldwide [9]. The number is much more than active smokers alone. This health issue would inevitably pose a big burden on our health care system.

Although the relationship between passive cigarette smoking and osteoporosis is established, the underlying mechanisms for such association are not defined. Indeed, it is hard to delineate whether there is a direct causal relationship between smoking and osteoporosis, since numerous confounding factors existed in humans that cannot be controlled in clinical studies, such as alcohol consumption, dietary, and steroid intakes and also the extent of physical activities in these subjects [10]. With these limitations, experimentation in animals could be very helpful in understanding the pathogenesis of smoking-induced bone loss. It is unethical to do any prospective and randomized trials in humans to force humans to smoke while excluding other confounding factors in the same groups of subjects. Various passive smoking mouse models were developed and widely used for studying the effects of cigarette smoking in different organs [11–17]. Existing study on smoking and bone loss in mice only showed bone

biomechanical properties changes without clues on the possible underlying mechanisms [18]. Although bone loss in spine was found in one study, direct inhalation of smoke through nostrils and confinement in a limited space may produce psychological stress to animals. This may produce adverse effects on bones as well [19].

To this end, the aim of the current study is to use a relatively non-stress and clinically relevant animal model of passive smoking and demonstrate the relationship between passive cigarette smoking and bone loss, and further understand the underlying mechanisms. The current mouse passive smoking model used in this study was well established for investigating the effect of smoking in gastrointestinal tract for over 10 years [20, 21]. This setup is simple but it can standardize the flow rates of both smoke and ambient air pumping into a close environment. This setup is very much like those situation found in humans in a confined room with people smoking passively. It was also confirmed that minimal stress was created to animals in this smoking model and also the blood nicotine level was comparable to moderate and heavy smokers [22], making it a good model simulating the actual condition of cigarette smoke exposure in humans. Besides, in order to study the direct effects of cigarette smoke on osteoblasts and osteoclast, MC3T3-E1 and RAW 264.7, the mouse preosteoblast and macrophage cell lines, respectively, were also used in this study. Since thousands of chemical species were produced from ignited cigarettes [23], it is inappropriate and also unpractical to investigate the effects of individual component or even groups of chemicals from cigarette smoke on bone cells. Therefore, the effects of sera from mice exposed to either normal ambient air or to cigarette smoke, on cell viability and differentiation on these bone cells, were assessed, in order to fully delineate the mechanisms of smoking-induced osteopenia in animals.

## Materials and Methods

### Animals

Sixty female BALB/c mice, aged 10-week-old, were supplied by the Laboratory Animal Service Center, The Chinese University of Hong Kong (CUHK). The mice were housed under a 12-h light–dark cycle at the temperature of 22 °C in cages (18.5 × 38 × 24 cm). Eight mice were kept in one cage according to their assigned groups. Teklad Global 18 % Protein Extruded Rodent Diet (Harlan Laboratories) with 1 % calcium was used as conventional diet. The mice had free access to food and water.

## Cigarette Smoke Exposure

The experimental setup was described according to the one used in previous studies [20, 24], with different concentrations of smoke exposure. Mice were randomly assigned into three groups: control group, 2 % smoking group, and 4 % smoking group, with eight mice in each group. The 2 % smoking group and 4 % smoking group were exposed to 2 % (v/v) and 4 % (v/v) cigarette smoke. The concentration of nicotine in the serum of the mice after smoking was proven to be similar to the levels in smokers in humans after smoking as high as 0.1 ng/ $\mu$ l in arterial circulation of chronic smokers [20, 22, 25]. They were put into a ventilated acrylic smoke chamber (18 × 39 × 24 cm) before smoke exposure began. Commercial cigarettes Good Companion (Philip Morris Products, Switzerland), with 1.2-mg nicotine and 13-mg tar per cigarette, were used in this study. The unfiltered cigarette smoke and atmospheric air were sent into the chamber simultaneously by two peristaltic pump at a constant rate of 20 ml/min smoke with 980 ml/min air, or 40 ml/min smoke with 960 ml/min air. The tubings delivered cigarette smoke were cleaned, and the flow rate was calibrated every week to ensure the consistency of flow for cigarette smoke. Mice passively smoked for 1 h per day, 6 days per week. The smoking period totally lasted for 14 weeks. The control group was given atmospheric air instead of cigarette smoke in the same condition as the smoking groups. Mice were then sacrificed at the end of the whole smoking period.

## Urine Cotinine Level

Urine was collected every 2 weeks. The total 24-h urine output from four mice in each group was pooled together in a metabolic cage. The urine was kept at  $-80^{\circ}\text{C}$  for further determination of cotinine concentration using a Calbiotech cotinine ELISA kit (catalogue no. CO096D). Data from week 4 to week 14 of the same group were grouped and analyzed.

## Urine Calcium, Phosphorus, and DPD Content

Calcium and phosphorus concentrations in urine were determined using Stanbio commercial kits Calcium (CPC) LiquiColor<sup>®</sup> Test (catalogue no. 0150-350) and Phosphorus Liqui-UV<sup>®</sup> Test (catalogue no. 0830-125). Urine DPD level was assessed by the Quidel MicroVue tDPD ELISA kit (catalogue no. 8032). Results were normalized by the concentration of creatinine in urine. Creatinine concentration was measured by a commercial kit Stanbio Direct Creatinine LiquiColor<sup>®</sup> Test. All procedures were done according to the protocols provided by the manufacturer. In

order to get enough sample size for statistical analysis, the whole 14-week smoke exposure experiment and the collection of urine were repeated using the control and 4 % smoking groups, with eight mice in each group.

## Serum ALP Activity

Sera were collected every 2 weeks. About 0.2-ml blood samples were drawn from the orbital sinus of mice each time with isoflurane as anesthetic to the animals. They were allowed to clot and centrifuge at  $4^{\circ}\text{C}$  at 3000 rpm for 15 min. The supernatants were collected and stored at  $-80^{\circ}\text{C}$  for further biochemical assay. At the end of the experiment, animals were anesthetized by ketamine and xylazine mixture and sacrificed by heart puncture using a 25G needle. About 0.8 ml of whole blood was collected and allowed to clot for 30 min. They were centrifuged at 3000 rpm for 15 min. The supernatants were collected and stored at  $-80^{\circ}\text{C}$ . The Stanbio Alkaline Phosphatase LiquiColor<sup>®</sup> Test kit was used to determine the activity of ALP in these mouse sera according to the protocol provided by the manufacturer.

## Micro-computed Tomography

After euthanization of the animals, their left femora were excised and scanned with a high-resolution cone-beam micro-tomographic system ( $\mu$ CT40; Scanco Medical AG, Switzerland). The scanning was conducted at 70 kVp and 114  $\mu$ A with a resolution of 12  $\mu$ m per voxel for 400 consecutive sections proximal from femoral condyle. The microstructure of bones was reconstructed three-dimensionally by the build-in software of the machine. Region of interest of the trabecular bone was identified with semi-automatically drawn contour at each two-dimensional section. The volume of interest for trabecular bone within the distal femoral metaphysis was determined by 200 continuous slices starting from the growth plate at the distal epiphysis toward the proximal region. Segmentation parameters were evaluated at: Sigma = 1.0, Support = 1.0, and Threshold = 150. Parameters from direct model (Bone volume ( $\text{mm}^3$ ), trabecular number (Tb. N.), trabecular thickness (Tb. Th.), and trabecular plate separation (Tb. Sp.), bone volume density (BV/TV) and also the structural model index (SMI)) were finally analyzed.

## Preparation of Sera from Mice for In Vitro Studies

Control group or smoking group was exposed to ambient air or 4 % cigarette smoke respectively by the same method as described before. After 2 weeks of smoking, mice were sacrificed and serum samples were collected

within 2 h after the last smoking session by heart puncture. The sera from the animals in the same group were pooled together to make up the stocks of control and 4 % smoking groups. They were filtered through the 0.22- $\mu$ m low protein binding cellulose acetate syringe filter (IWAKI, Japan). The sera were aliquoted and stored in  $-80^{\circ}\text{C}$  for later use in cell studies.

### Cell Culture

Mouse macrophage and osteoblastic cell lines, RAW 264.7, and MC3T3-E1 (subclone 4) were purchased from ATCC. They were maintained in DMEM and  $\alpha$ MEM, respectively, with 10 % heat-inactivated FBS, 100 units/ml penicillin, and 100- $\mu$ g/ml streptomycin, under 5 %  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . Only those cells under 35 passages were used for the studies.

### MTT Cell Viability Assay

RAW 264.7 cells were seeded at  $1 \times 10^3$  cells/well, and MC3T3 were seeded at  $5 \times 10^3$  cells/well onto 96-well plates. After 24 h of incubation for adherence, cells were then treated without or with the serum either from the control or 4 % smoking mice at a concentration of 1 % for 72 h. Medium was aspirated and replaced with 100- $\mu$ l DMEM or  $\alpha$ MEM, respectively, with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at a concentration of 0.5 mg/ml. It was further incubated in  $37^{\circ}\text{C}$  for 3 h and the medium was removed. DMSO was added to the wells, and the optical density at 570 nm was measured in a microplate spectrophotometer.

### Osteoclast Differentiation and TRAP Staining

RAW 264.7 cells were seeded at  $1 \times 10^3$  cells/well onto a 96-well plate and incubated for 24 h. The medium was then replaced with  $\alpha$ MEM containing 50  $\mu$ g/ml ascorbic acid. To induce osteoclast differentiation, human recombinant receptor activator of NF- $\kappa$ B ligand (RANKL) (50 ng/ml) was added and the cells were then treated with the serum at a concentration of 1 % from either the control or 4 % smoking mice for 72 h. Cells without treating RANKL and mouse serum served as a negative control. Tartrate-resistant acid phosphatase (TRAP) staining was performed by using a TRAP staining kit (Sigma, USA) according to the protocol provided. Briefly, the cells were washed by PBS and then fixed. After washing with distilled water, TRAP staining reagent was added to each well and incubated for 1 h at  $37^{\circ}\text{C}$ . The cells were washed in distilled water and observed under microscope. Only cells with more than three nuclei and stained with TRAP-positive were counted as differentiated osteoclasts.

### Osteoblast Differentiation, ALP Assay, and Calcium Deposition Assay

MC3T3 cells were seeded at  $4 \times 10^4$  cells/well onto a 12-well plate and incubated for 72 h. The cells were fasted for 24 h and then replaced with an osteogenic medium ( $\alpha$ MEM with 10 % FBS, 50- $\mu$ g/ml ascorbic acid and 5- $\mu$ M glycerol 2-phosphate) to induce osteoblast differentiation. They were treated with serum at a concentration of 1 % either from the control or 4 % smoking mice for 7, 14, 21, 28 days. The osteogenic medium and the mouse serum were replaced every 3 days.

After the treatment for 7, 14, 21, 28 days, cells were washed with PBS twice and lysed with 200- $\mu$ l lysis buffer (50 mM Tris, 2 mM  $\text{MgCl}_2$ , 0.1 % Triton X-100, pH 7.4) in two freeze-thaw cycles. It was then centrifuged at  $20,000 \times g$ , and the supernatant was assayed for ALP activity using Stanbio Alkaline Phosphatase LiquiColor<sup>®</sup> Test kit by the same method mentioned above. The ALP activity was normalized by the protein concentration which was determined by the Bradford protein assay. The cell lysate was dried in an oven overnight and the residue was dissolved in 0.6 N HCl for 24 h. The concentration of calcium was determined using the Stanbio Calcium (CPC) LiquiColor<sup>®</sup> Test. The acid was dried and the residue was dissolved in 200- $\mu$ l protein lysis buffer (0.1 M NaOH, 0.1 % SDS). The amount of calcium was normalized by the protein concentration. The MC3T3 cells were treated for 21 and 28 days and washed with PBS twice and fixed by neutral buffered formalin. Alizarin Red S stain (2 g/ml, pH 7.4) was added to the fixed cells for 30 min. Excessive stain was washed away by distilled water.

### Statistical Analysis

All results are expressed in mean  $\pm$  SEM, and  $p$  value  $< 0.05$  is considered as statistically significant. One-way or Two-way ANOVA with Bonferroni post test were used for statistical analysis unless otherwise stated in GraphPad Prism 5 software.

## Results

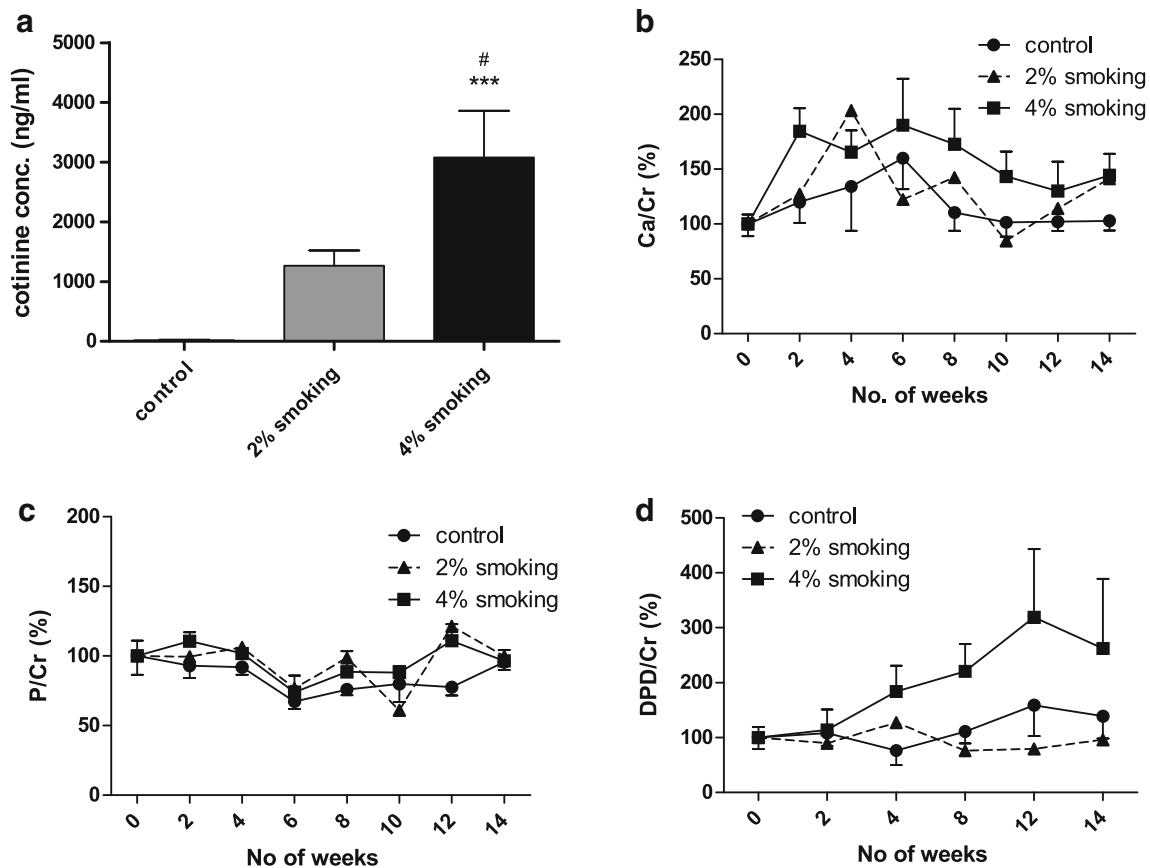
### Urine Cotinine Level

The urine cotinine level of the control group without cigarette smoking was  $20.71 \pm 17.24$  ng/ml. This observation agrees with the previous reports in which that the cotinine level was always lower than 100 ng/ml in non-smokers [26]. For the 2 and 4 % smoking groups, the urine cotinine concentrations were concentration-dependently

increased to  $1264 \pm 883$  and  $3076 \pm 2722$  ng/ml, respectively in these animals (Fig. 1a).

#### Urine Calcium, Phosphorus, and DPD Contents

The urine calcium (Ca), phosphorus (P), and DPD concentrations were normalized by the concentration of creatinine (Cr) levels in the mice. The value at week zero was normalized in each group as 100%. The values of other time points were compared to week zero. Results showed that there were no significant changes in urine Ca/Cr ( $p = 0.0937$ ) and P/Cr ( $p = 0.0854$ ) after smoking from week 0 to week 14 (Fig. 1b and c). Although there was no significant difference at individual time point, the trend of DPD/Cr was significantly different from the control group (Fig. 1d). The two-way ANOVA analysis results showed that smoking accounted for 12.27% of the total variance in DPD/Cr ( $p = 0.0147$ ).



**Fig. 1** The urine chemical properties of the mice after passive cigarette smoking at different concentrations. **a** Urine samples collected from week 4–14 in each group were pooled and cotinine level was analyzed by ELISA ( $n = 12$  in each group). The changes in **b** calcium, **c** phosphorus, and **d** deoxypyridinoline (DPD) level in urine throughout the smoking period. Urine samples collected from week 0–14 in each

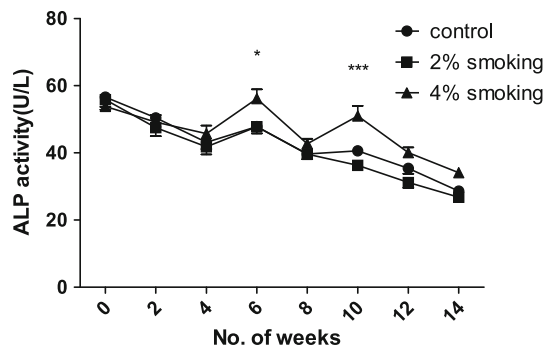
#### Serum ALP Activity

The total serum ALP activity decreased in a time-dependent manner in all groups during the experimental period. The ALP activity of 4% smoking group was significantly higher than the control group in week 6 and 10, in contrast to 2% smoking group, which had similar levels comparing to the control group (Fig. 2a). In the two-way ANOVA analysis, smoking accounted for 6.06% of total variance ( $p < 0.0001$ ) and it had significant correlation with time ( $p = 0.0068$ ). This finding indicated that the bone resorption rate in the heavy smoking group kept at a high level throughout the whole smoking period. However, moderate smoking at 2% of cigarette smoke did not produce this effect.

#### Micro-computed Tomography

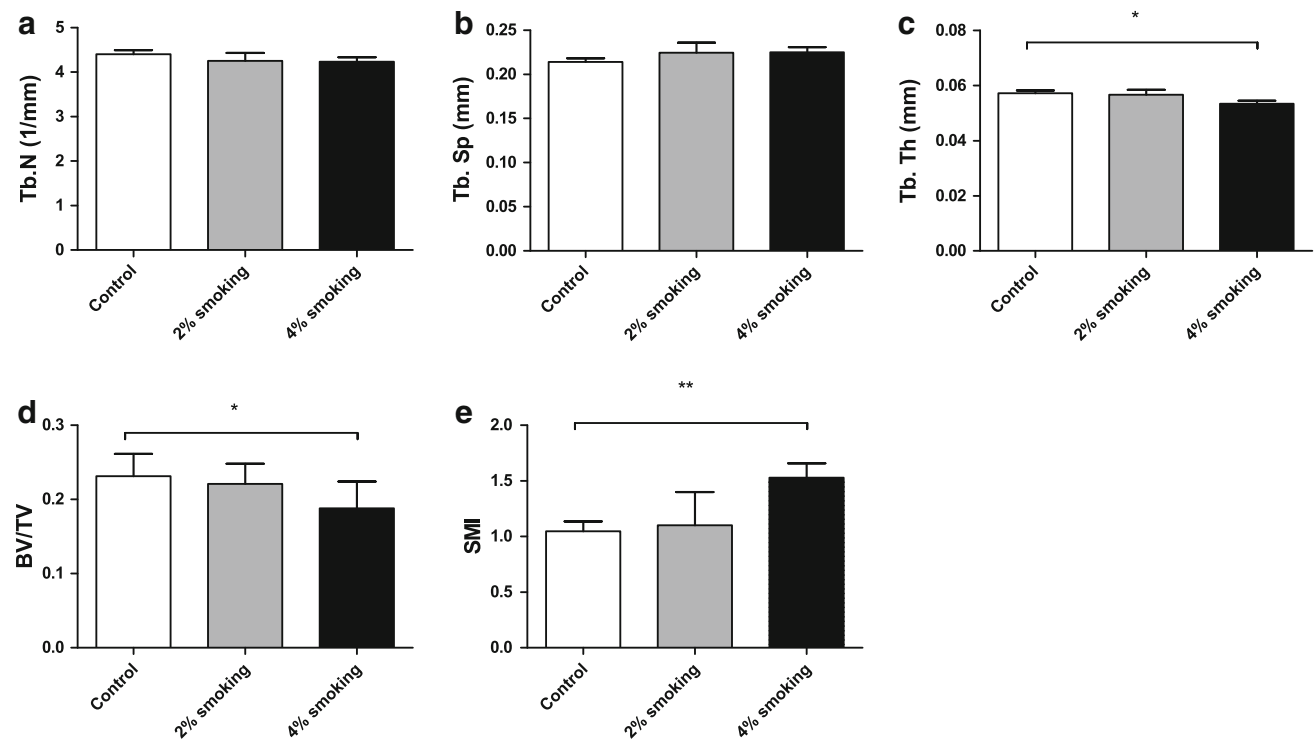
The number and spacing of trabecular bone remained unchanged after cigarette smoking, but the trabecular

group were assessed by biochemical assays and ELISA. All samples were normalized by concentration of creatinine (Cr) and the level at week 0 in each group was assigned as 100% ( $n = 4$  in each group, except  $n = 2$  in 2% smoking group). Significant difference: \*\*\* $p < 0.001$  for difference from the control without cigarette smoking; # $p < 0.05$  for difference from 2% smoking group



**Fig. 2** Bone turnover marker in serum throughout the smoking period. The total ALP activity of serum samples collected from week 0–14 in each group was measured ( $n = 8$  in each group, except  $n = 7$  in 2 % smoking group). Significant difference: \* $p < 0.05$ ; \*\*\* $p < 0.001$  for difference between 4 % smoking group and the control group

thickness was significantly reduced by 6.7 % in the 4 % smoking group ( $p = 0.026$ ). The BV/TV was also significantly decreased by 18.6 % after 4 % cigarette smoking ( $p = 0.021$ ). The SMI, which is a parameter describing the structure of trabecular bone, was significantly elevated by 46 % in the 4 % smoking group ( $p = 0.008$ ). There was no difference in all the parameters measured when comparing between the 2 % smoking and the control groups (Fig. 3).



**Fig. 3** The micro-computed tomography of mouse distal femora. **a** Trabecular number (Tb. N.). **b** Trabecular thickness (Tb. Th.). **c** Trabecular spacing (Tb. Sp.). **d** Relative bone volume (BV/TV), and **e** structure model index (SMI) of distal femur of mice after smoking

### MTT Cell Viability Assay

There was no significant change in cell viability after treating the MC3T3 cells with or without the mouse serum, but the RAW 264.7 cells had a significant increase in cell viability after incubation with the serum either from the control or 4 % smoking groups of mice. However, there was no significant difference in cell viability between the two groups in both RAW264.7 and MC3T3 cells in vitro (Fig. 4).

### RAW 264.7 Cells TRAP Staining

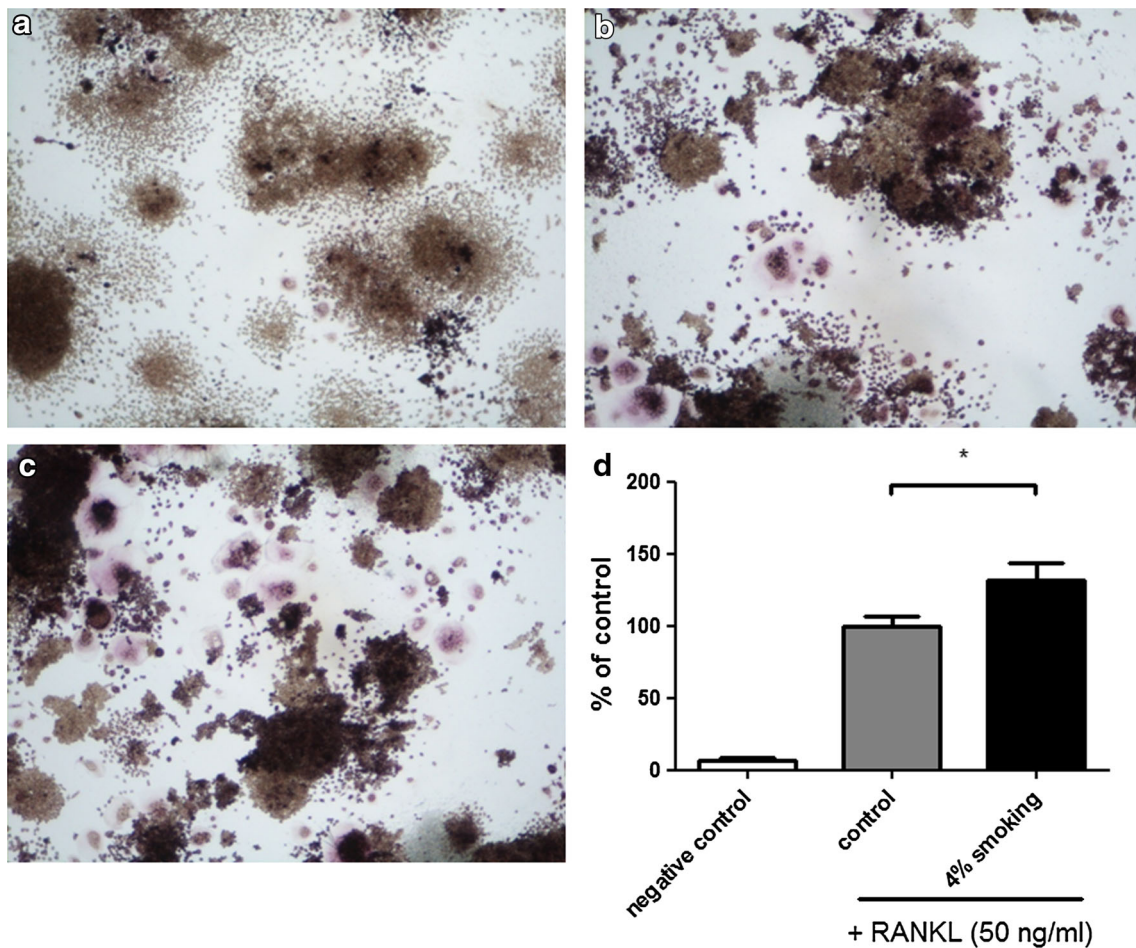
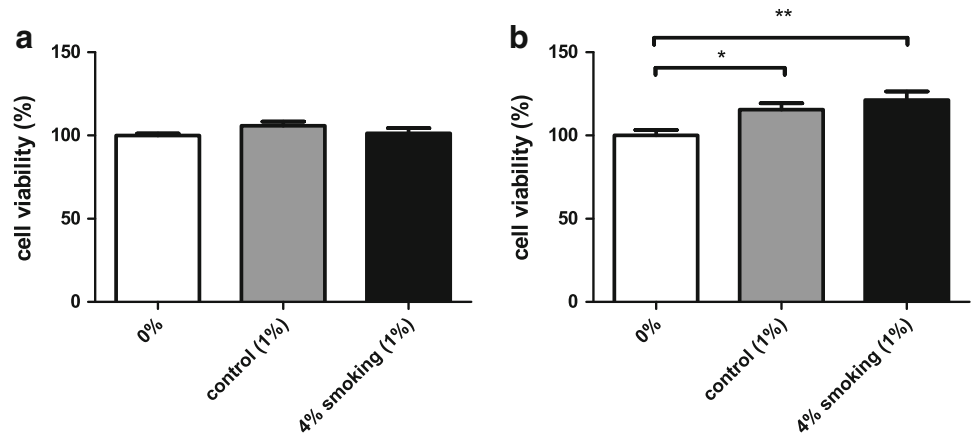
The number of mature osteoclasts was significantly increased after treating the cells with RANKL. Incubation with the serum from the 4 % smoking group significantly increased the number of TRAP-positive multinucleated cells by 32 % when compared with the control group (Fig. 5).

### MC3T3 Cells ALP Assay and Calcium Deposition Assay

The ALP activity was significantly reduced after treating the MC3T3 cells with the serum from 4 % smoking mice at

for 14 weeks were scanned and measured ( $n = 8$  in each group, except  $n = 7$  in 2 % smoking group). Significant difference: \* $p < 0.05$  for difference from control group

**Fig. 4** Cell viability of **a** MC3T3 and **b** RAW 264.7 after treating the cells with 1 % serum from 4 % smoking or control mice for 72 h by MTT assay ( $n = 6$  in each group).  $*p < 0.05$ ;  $**p < 0.01$  for difference from cells without treating mouse serum



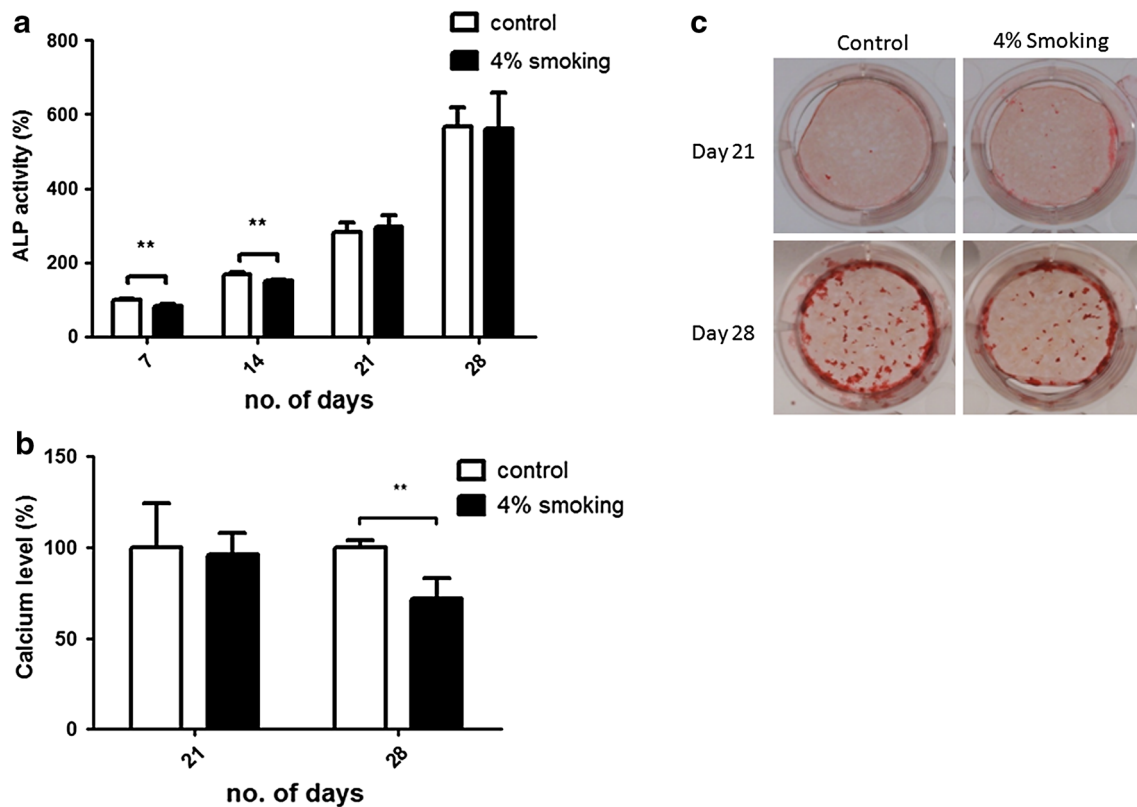
**Fig. 5** Assessment on osteoclast differentiation by TRAP staining on RAW 264.7 cells **a** without RANKL and mouse serum, **b** with 1 % control mouse serum and 50 ng/ml RANKL, and **c** with 1 % smoking

mouse serum and 50 ng/ml RANKL. **d** The statistics of TRAP + multinuclei cells count in each well of 96-well plate ( $n = 18$ ).  $*p < 0.05$  for difference from cells treating with control mouse serum

7 and 14 days. This effect was diminished at day 21 and 28 (Fig. 6a). However, the amounts of bone nodule and calcium formation in the 4 % smoking group was significantly reduced by 28 % at day 28 when compared to the control group (Fig. 6b).

**Discussion**

Active smoking is defined as voluntary smoking directly from the ignited cigarette. Such experimental condition is almost impossible to be reproduced in any animal setting.



**Fig. 6** Assessment on osteoblast differentiation and bone formation function **a** ALP assay was done on MC3T3 cells treated with control or 4 % smoking mouse serum and the ALP activities at day 7, 14, 21, 28 were measured. ALP activity of control group at day 7 was assigned as 100 % ( $n = 6$ ). **b** The amount of calcium deposited during osteoblast differentiation in MC3T3 cells treated with control

or 4 % smoking mouse serum. **c** Alizarin red S staining was done on MC3T3 cells treated with control or 4 % smoking mouse serum at day 21, 28. Calcium nodules formed were stained red in color ( $n = 4$ ). Significant difference:  $**p < 0.01$  for difference from cells treating with control mouse serum (Color figure online)

For this reason, our study applied a passive smoking animal model. This can mimic the environment where humans are involuntarily exposed to cigarette smoke, e.g., in the designated smoking areas of the public and also at home where family member(s) are smokers. The number of people being affected could be enormous. Furthermore, second-hand smoke exposure was also associated with increased risk of osteoporosis [8]. It is reasonable to use a passive smoking model to study the effects of cigarette smoking on bone metabolism and structures. In addition, mouse would be a better model comparing to rat for further investigation on genetic association in the mechanistic study due to well-known genetic profile and well developed transgenic technology in mouse [27]. It is suggested that the Balb/c strain is a good animal model for studying bone aging since it exhibits changes that can be seen in humans, showing that it is a clinically relevant animal model [28]. In another study, greater bone loss to ovariectomy was induced at various sites in Balb/c strain when comparing to the C57BL/6J strain of mice [29]. It is an appropriate strain of mice studying osteoporosis in animals.

This study uses a mouse passive cigarette smoking model to demonstrate detrimental effects on bone metabolism and microstructure in mice. However, cigarette smoking in the current study did not produce any observable toxicities in lung and liver by histological analysis and enzyme assays such as ALT and AST activities (data not shown). The body weight and food consumption were similar among all treatment groups. Their physical activities were similar in all groups, suggesting that bone could be one of the first targeted organs to be affected by cigarette smoking. Previous studies showed that using the similar smoking model, the blood nicotine concentration attained a comparable level with humans after cigarette smoking. This was also proportional to the number of cigarettes smoked daily [20, 22, 25]. Our present study also verified that the urine cotinine level, the major metabolite of nicotine, was also proportional to the number of cigarettes smoked in mice. Moreover, the urine cotinine level in the 2 % smoking group was similar to those in moderate smokers ( $\leq 20$  cigarettes per day). Likewise, the level in the 4 % smoking group was similar to heavy smokers ( $>20$



cigarettes per day) [30]. Only animals with 4 % cigarette smoking showed detrimental effects on bones after smoking for 14 weeks, which is comparable to about 12 years in human lifespan.

In order to determine the effect of heavy smoking in the trabecular bone structure of distal femur,  $\mu$ -CT analysis was performed. The 3D  $\mu$ -CT images from the heavy smoking group showed significant bone volume (BV/TV) reduction. Inhalation of cigarette smoke similar to the smoke inhaled by heavy smokers also resulted in a significant increase in SMI, indicating that the bone microstructure altered from a more plate-like structure to a more rod-like structure, which is less rigid and less capable of withstanding mechanical stress from various directions [31]. The reduction of bone volume was mainly due to the decrease of trabecular bone thickness (Tb. Th.). The urinary excretion of calcium was also increased in the 4 % smoking group, and this finding agrees with the reduction of BV/TV in our study. The morphological changes after cigarette smoking in the current study are also in accord with various clinical reports stating that smokers have lower BMD with higher risk of osteoporosis and bone fracture [5, 32, 33]. Some meta-analyses also suggest that, this effect is probably due to the long-term cigarette smoking but not to other confounding factors as reported in human subjects [34]. Our animal study fully affirms this hypothesis and showing that cigarette smoking can indeed induce osteopenic condition in the absence of other confounding factors, like alcohol drinking, diet, physical activities or steroid intake as found in humans.

Type-I collagen plays a predominant role in bone mineralization process in a way that it serves as a template for the nucleation and deposition of calcium phosphate crystals [35]. The urinary excretion of DPD, the bone-specific collagen crosslinking peptide, was augmented after 4 % cigarette smoking. It may be due to the outcome of increased degradation of collagen in bone. The current mouse smoking model could give us clues that collagen metabolism might be adversely affected by heavy cigarette smoking.

The 10 week-old mice used resembles the situation in humans that smoking among adolescence in females with higher growth rate in bone. In our study, the serum total ALP activity was found to be decreased with time. This finding is similar to the trend found in women, in which the ALP activity decreases during growth in the early ages of their lives [36]. However, when bone is degraded, the ALP from osteoblasts is released into the blood stream and thus ALP activity in serum elevates. Our data demonstrated that the effect of 4 % cigarette smoking on ALP became prominent at week 6 and 10, which implied that bone turnover activities were increased starting from week 6. However, the effect was slowing down during the later part

of the experiment. Unlike age-related estrogen deficiency-induced osteopenia, which is a relatively constant physiological change, cigarette smoke may induce temporal changes of ALP leading to uncontrolled bone turnover events at different period of time in smoking. A significant higher ALP activity in the 4 % smoking group would further confirm that the bone turnover rate was indeed increased by smoking.

There were only a few similar studies to investigate the effects of cigarette smoking on bone. In one of these studies growing female mice smoking passively for 12 weeks their femoral biomechanical properties were slightly defected [18]. Our data showed a stronger deteriorating effect in BV/TV and Tb. Th. from the  $\mu$ CT findings. Nevertheless, this study did not assess the biochemical changes in these animals. In a separate study using  $\mu$ CT technology it was found that both vertebral BV/TV and Tb. Th. were decreased significantly after smoking for 6 months in mice [19]. One study indicated that bone resorption marker tartrate-resistant acid phosphatase (TRAP) 5b was increased while bone formation marker bone-specific alkaline phosphatase in serum was decreased after passive smoking for 4 months [37]. Additionally our study used an important bone turnover marker, the total ALP to reflect bone turnover rate. It was found to be significantly increased after smoking indicating that the bone turnover rate was enhanced. Moreover, we also measured the mineral contents, like calcium and phosphorus, and also collagen content DPD in urine. Our study also showed for the first time that the collagen metabolism in bone was altered after cigarette smoking.

Seropharmacology is a technique to study the pharmacology of special serum on cells. The idea of this technique was firstly published in 1987 [38]. Through this technique, direct observation of the pharmacological effects of the passive smoke components on cells becomes feasible. The in vitro results from seropharmacological studies are more physiologically relevant and representative to those found in whole animals. This approach is much better than those studies using either smoke extracts or chemicals derived from smoke, in order to explain the in vivo outcome of cigarette smoking on bone. In the current study, sera from smoking and control mice were used to investigate the effect of smoking on bone cells. This experimental setup could reflect the actual and direct effects of cigarette smoking on bone cells. As the major smoke components, e.g., nicotine and other chemical compounds in smoke, after inhalation would have been quickly metabolized into different active metabolites which are probably the major culprits to different diseases including osteoporosis in humans. Studying the direct actions of sera from active smokers on bone cells instead of investigating a single component like nicotine, or chemical extracts from

cigarette smoke [39] may reflect the real situation of cigarette smoking on bone structure and metabolism. Examining a single compound or even a complex of chemicals derived from cigarette smoke cannot represent cigarette smoking which is complex in nature, as well as numerous metabolic reactions should occur in the body after inhalation of smoke. These reactions would have modified the xenobiotic composition and also modulate endogenous reactions in the body. Treating the cells with mouse serum would therefore be an appropriate approach to reflect the real action of cigarette smoking since it mimics the physiological condition in which the bone cells supposed to be exposed to only after inhalation of smoke in the body. Indeed, results from the current *in vitro* study showed that cigarette smoking could directly affect the differentiation processes and function of both osteoblasts and osteoclasts.

At the early stage of osteoblast differentiation, the ALP activity, a marker of osteoblast differentiation and in early bone formation process, was reduced in 4 % smoking group. Besides, reduction of bone nodules formation in the later stage was also observed in the 4 % smoking group, providing a direct evidence that cigarette smoking indeed can retard bone formation and this could result from the hindrance of the osteoblast differentiation process. However, alteration in ALP activity diminished in the later time points of the experiment, implying that other endogenous factors involving in the osteoblast differentiation in the body may be activated by smoking as a feedback mechanism in deterring the action of cigarette smoking.

Cigarette smoking did not only alter the differentiation of osteoblasts, but also enhanced osteoclast differentiation from macrophages under the action of RANKL, an essential inducing agent for osteoclast differentiation [40]. TRAP + multi-nucleated cell as an indicator of mature osteoclast is the exclusive cell type for bone resorption. Therefore an increased number in mature osteoclasts may indicate enhanced bone resorption. In addition to these results, no difference was found in cell viability between control and 4 % smoking group on both MC3T3 and RAW264.7, showing that the effects were independent of cell viability but it merely acted on differentiation pathways involved in osteoblasts and osteoclasts maturation. The data from *in vitro* study also give us evidence that cigarette smoking on the one hand can promote the formation of osteoclasts and on the other hand it can inhibit the function of osteoblasts in bone formation. All these correlate well with our findings from the mouse smoking model that the bone microstructure was deteriorated after heavy smoking.

There are some limitations for this research study. Firstly, the collection of urine was performed once every 2 weeks. However, it could have been done more

frequently in order to increase the accuracy of the data. Besides, the cell culture systems only demonstrate the direct effect of smoking either on osteoblasts or osteoclasts. These systems however did not show the effects of cigarette smoking on the interaction between the two types of bone cells. Both of which concurrently play an important role in bone remodeling process in the body [41]. Further experiments to examine osteoblast and osteoclast numbers in bones from the smoke-exposed animals would have provided more evidence to reveal that interaction between bone formation and degradation.

Furthermore, the detailed composition of the serum used in the *in vitro* studies has not been characterized in detail for the types of metabolites from various smoke toxic products that might be present. The composition of serum with respect to toxic products and differences from normal serum should be analyzed in future.

In conclusion, the current passive mouse smoking model successfully induced alteration in bone microstructure in femur and increased bone turnover in animals. The results resemble clinical data to affirm the direct detrimental effects of cigarette smoking on bone structures and metabolism. These adverse actions are further confirmed in serum from cigarette smoking animals. It could act directly on osteoblast and osteoclast differentiations and their functions. Further study is required to explain the detailed mechanisms of action of how this serum acts on bone cells.

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**Conflict of interest** Chun Hay Ko, Ruby Lok Yi Chan, Wing Sum Siu, Wai Ting Shum, Ping Chung Leung, Lin Zhang, and Chi Hin Cho declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** All the experimental procedures had been approved by the Animal Experimentation Ethics Committee (CUHK) in accordance to the Department of Health (HKSAR) guidelines in Care and Use of Animals.

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