Heated Tobacco Products Impair Cell Viability, Osteoblastic Differentiation, and Bone Fracture-Healing

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Background: The negative impact of cigarette smoking on bone union has been well documented. However, the impact of heated tobacco product (HTP) use on bone fracture-healing remains unclear. The present study investigated the effect of HTPs on preosteoblast viability, osteoblastic differentiation, and fracture-healing and compared the effects with those of conventional combustible cigarettes.

Methods: Cigarette smoke extracts (CSEs) were generated from combustible cigarettes (cCSE) and HTPs (hCSE). CSE concentrations were standardized by assessing optical density. Preosteoblast (MC3T3-E1) cells were incubated with normal medium, cCSE, or hCSE. The cell viability was assessed via MTT assay. After osteoblastic differentiation of CSE-exposed cells, alkaline phosphatase (ALP) activity was assessed. To assess the in vivo effects of CSEs, a femoral midshaft osteotomy was performed in a rat model; thereafter, saline solution, cCSE, or hCSE was injected intraperitoneally, and bone union was assessed on the basis of micro-computed tomography (μCT) and biomechanical analysis 4 weeks later.

Results: MC3T3-E1 cell viability was reduced in a time and concentration-dependent manner when treated with either cCSE or hCSE. ALP activity after osteoblastic differentiation of cCSE-treated cells was significantly lower than that of both untreated and hCSE-treated cells (mean and standard deviation, 452.4 ± 48.8 [untreated], 326.2 ± 26.2 [cCSE-treated], and 389.9 ± 26.6 [hCSE-treated] mol/L/min; $p = 0.002$). Moreover, the levels of osteoblastic differentiation in untreated and hCSE-treated cells differed significantly ($p < 0.05$). In vivo assessment of the femoral midshaft cortical region revealed that both cCSE and hCSE administration significantly decreased bone mineral content 4 weeks after surgery compared with levels observed in untreated animals (107.0 ± 11.9 [untreated], 94.5 ± 13.0 [cCSE-treated], and 89.0 ± 10.1 mg/cm^3 [hCSE-treated]; $p = 0.049$). Additionally, cCSE and hCSE-exposed femora had significantly lower bone volumes than unexposed femora. Biomechanical analyses showed that both cCSE and hCSE administration significantly decreased femoral maximum load and elastic modulus ($p = 0.015$ and 0.019).

Conclusions: HTP use impairs cell viability, osteoblastic differentiation, and bone fracture-healing at levels comparable with those associated with combustible cigarette use.

Clinical Relevance: HTP use negatively affects bone fracture-healing to a degree similar to that of combustible cigarettes. Orthopaedic surgeons should recommend HTP smoking cessation to improve bone union.

A wide variety of tobacco products, including combustible cigarettes, electrical cigarettes, and heated tobacco products (HTPs), are available worldwide. Combustible cigarettes, which have the longest history and remain most popular, have been associated with various harmful effects to the skeletal system and overall health[1,2]. As global combustible cigarette sales have been declining, the tobacco industry has rapidly been marketing new products, including HTPs[3].

Heated tobacco smoking technology is based on a unique electronic method of heating, which generates aerosols from tobacco sticks. Tobacco-heating systems operate at lower temperatures (240°C to 350°C) than conventional cigarettes (>600°C) and produce lower levels of harmful chemicals such as tar than conventional cigarettes[4]. HTP use has been increasing, especially in several developed countries such as Japan and Italy[5-9]. In the United States, the Food and Drug Administration authorized the marketing of HTPs in 2019. Due to the expected shrinking of

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the e-cigarette market in the near future, HTP consumption is predicted to increase rapidly[10,11].

Tobacco producers claim that HTPs are less harmful than traditional cigarettes[12-16]. They have performed multiple in vitro studies using human bronchial epithelial cells, coronary arterial endothelial cells, a 3-dimensional (3D) nasal culture model, gingival epithelial organotypic cultures, monocytic cells, and in vivo mouse models[17-22]. However, the World Health Organization questioned the safety of HTPs and warned consumers against their use[23]. Furthermore, to our knowledge, there have been no studies that have assessed the effect of HTPs on osteoblastic differentiation and fracture-healing. An independent investigation of the effects of HTPs that is not conducted by cigarette producers is needed.

We hypothesized that HTPs impair cell viability and osteoblastic differentiation but are less impactful than combustible cigarettes. Therefore, the purpose of this Japanese government-supported study was to investigate the effect of HTPs on cell viability, osteoblastic differentiation in vitro, and bone fracture-healing in vivo. Furthermore, findings for HTPs were compared with those for conventional combustible cigarettes with use of a cigarette smoke extract (CSE) method that allows researchers to standardize treatments.

Materials and Methods

CSE Preparation

CSEs from combustible cigarettes (cCSEs) and HTPs (hCSEs) were generated as previously described (Fig. 1)[24-27]. Briefly, combustible cigarette particulate matter was generated from commercially available cigarettes (Marlboro; Philip Morris) with 12 mg tar and 0.9 mg nicotine per cigarette with use of a puffing protocol of one 2-second puff per minute with a volume of 35 mL/puff under ISO3308 conditions. The filter was removed, and cigarettes were placed in a standard gas washing bottle with 25 mL of phosphate-buffered saline solution and were subjected to negative pressure with use of a plastic syringe. The resulting extract was filter-sterilized in 0.22-μm filter units (Sterile Millex-GS; Merck Millipore) before being used in the experiments. HTP particulate matter was obtained from a regular Marlboro HeatStick (Philip Morris). Smoke extract from HTP (hCSE) was generated with use of the same protocol that was used to create cCSE. The concentration of CSE generated from 1, 3, 6, and 9 cigarettes was determined and standardized by measuring the optical densities of materials at 320 nm (OD320) with use of a plate reader (Varioskan LUX; Thermo Fisher). Each CSE concentration was determined 3 times, and CSE from 3 cigarettes was defined as a 100% concentration of CSE[25,26]. The concentration of CSE from 3 cigarettes was measured at 320 nm to confirm that it was within a standard deviation (SD) of its expected value before being used in an experiment. To fairly assess the harmful effects, the numbers of cigarettes used to create cCSE and hCSE were matched. Solutions were diluted (1%, 5%, 10%, and 20% of original concentrations) and were immediately used for cell proliferation assays. An extract concentration that displayed no cytotoxicity was used to assess osteoblastic differentiation.

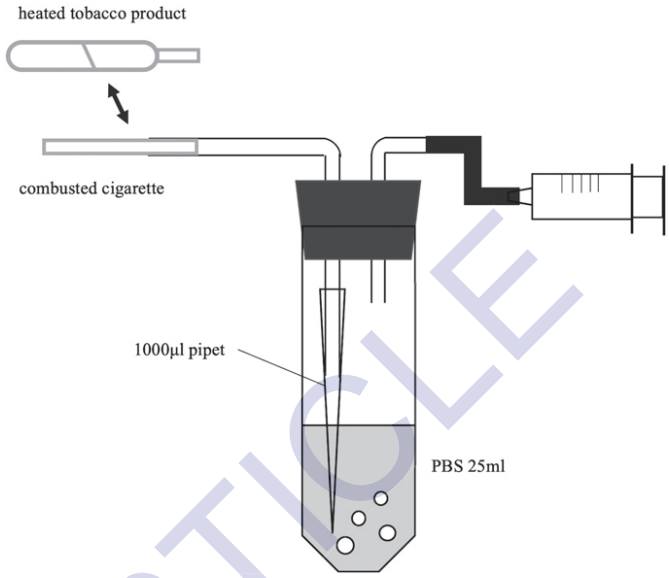

Fig. 1
Apparatus used for the preparation of cigarette smoke extract.

Cell Culture

The murine preosteoblast cell line MC3T3-E1 was purchased from the RIKEN cell bank. For maintenance of stock plates, MC3T3-E1 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Wako) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

In Vitro Assays

The viability of the MC3T3-E1 cells was assayed with use of an MTT Cell Proliferation/Viability Assay kit (R&D Systems) according to the manufacturer's specifications. Briefly, 1×10^4 cells were seeded onto a 96-well plate, and the medium was changed after 24 hours so that wells contained 0% to 20% CSE. After 8, 24, and 48 hours of incubation, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay was performed. Absorbance at 570 nm was determined with use of a microplate reader (U-3000 spectrophotometer; Hitachi). Six replicates for each group were assessed.

MC3T3-E1 cells were plated on a 24-well plate (5×10^4 cells/well) and were cultured with 300 ng/mL bone morphogenetic protein-2 (BMP-2) for 2 weeks to induce osteoblastic differentiation[28,29]. The culture medium was changed every 2 days while cells underwent osteoblastic differentiation. Cultured cells were treated as follows: 0% extract (control), 1% cCSE, or 1% hCSE.

After the stimulation of differentiation, alkaline phosphatase (ALP) activity tests and Alizarin Red S staining in MC3T3-E1 cells were carried out to evaluate early and late-phase osteoblastic differentiation[27,30]. ALP activity was assayed by measuring the optical density at 405 nm with use of Lab-Assay ALP (Wako Pure Chemical Industries). Alizarin Red S (Sigma-Aldrich) staining was performed to visualize calcium deposition. Briefly, cells were fixed with formalin, rinsed with deionized water, and stained with 0.5% Alizarin Red S solution.

Five random sections in each group were selected at 200× magnification, and the percentages of the areas positive for Alizarin Red S were quantified with use of ImageJ (National Institutes of Health) by an independent observer.

In Vivo Study

Animals and Treatments

Wild-type 8-week-old male Lewis rats (Japan SLC) were used for in vivo experiments. All animals were maintained with use of a 24-hour light-dark cycle, with food and water available ad libitum. All experimental animal procedures were approved by, and conducted in accordance with regulations of, the Osaka City University Graduate School of Medicine Committee on Animal Research (approval number: 19042). Rats were anesthetized by means of a subcutaneous injection of ketamine (50 mg/mL) (Sankyo) and xylazine (0.2 mg/mL) (Bayer HealthCare) at a ratio of 10:3 and a dose of 1 mL/kg body weight. All rats underwent surgical osteotomy to create a transverse fracture in the middle of the right femoral shaft. The fracture was fixed with intramedullary nailing with a Kirschner wire (1.2-mm diameter). A total of 18 rats were randomly divided into control, cCSE, and hCSE groups. All rats were injected intraperitoneally at days 0, 5, 10, 15, and 20 with 1 mL of phosphate-buffered saline solution, cCSE, or hCSE[31-33]. A 100% concentration of CSE was used. There were no exclusion cases.

Micro-Computed Tomography Assessment

Rats were killed via CO_2 asphyxiation 28 days postoperatively. Femora were collected and scanned with a micro-computed tomography (μCT) scanner (LaTheta LCT-100A; Hitachi-Aloka Medical). Sequential 240-μm slices of images with a resolution of 120 μm were used for calculations. The cortical region of the femoral midshaft was selected for analysis of microarchitectural parameters with use of LaTheta software (version 2.10; Hitachi-Aloka Medical). Parameters considered included bone mineral content and bone mineral density. Geometric parameters, including cortical thickness and cross-sectional area, were also analyzed. The trabecular bone region was not analyzed because of the insertion of the intramedullary nail. A total of 50 slices across the osteotomy line were used for the analysis of each animal. Scan data were reconstructed and were used to measure the volume of new bone with use of 3D image processing software (ExFact VR; Nihon Visual Science).

Biomechanical Assessment

At 4 weeks after surgery, 18 femora were used for biomechanical evaluation. A standardized 3-point bending test was performed in each group with use of a bending tester (EZ Graph; Shimadzu)[34,35]. Two parameters, maximum load (N) and elastic modulus (N/mm^2), were used to assess strength at the fracture site.

Statistical Analysis

All data are expressed as the mean and the SD. One-way analysis of variance with a post hoc t test was used to compare differences among the control, cCSE, and hCSE groups. The level of significance was set at $p < 0.05$. All analyses were performed with use of R (version 3.5.2; R Foundation for Statistical Computing) and EZR software (Saitama Medical Center, Jichi Medical University).

Source of Funding

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Results

Concentrations of cCSE and hCSE

Optical density increased in proportion with the number of cigarettes or HTPs added (Fig. 2). With use of OD320 values, both cCSE and hCSE concentrations could be reproducibly quantified. Based on a previous report, the current study adopted the optical density of cCSE and hCSE generated from 3 cigarettes and HeatSticks as 100% concentrations[25,26].

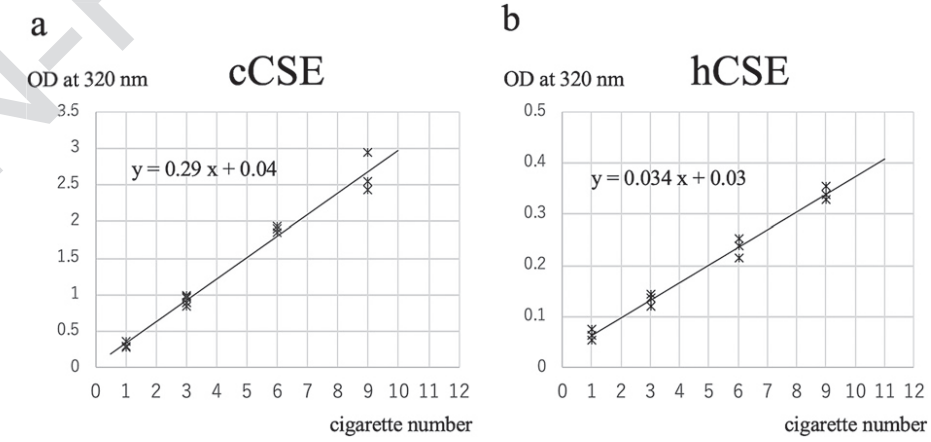

Fig. 2

Relationship between the number of cigarettes and the optical density (OD) of cigarette smoke extract. Values for **(a)** combustible cigarettes and **(b)** heated tobacco products are shown.

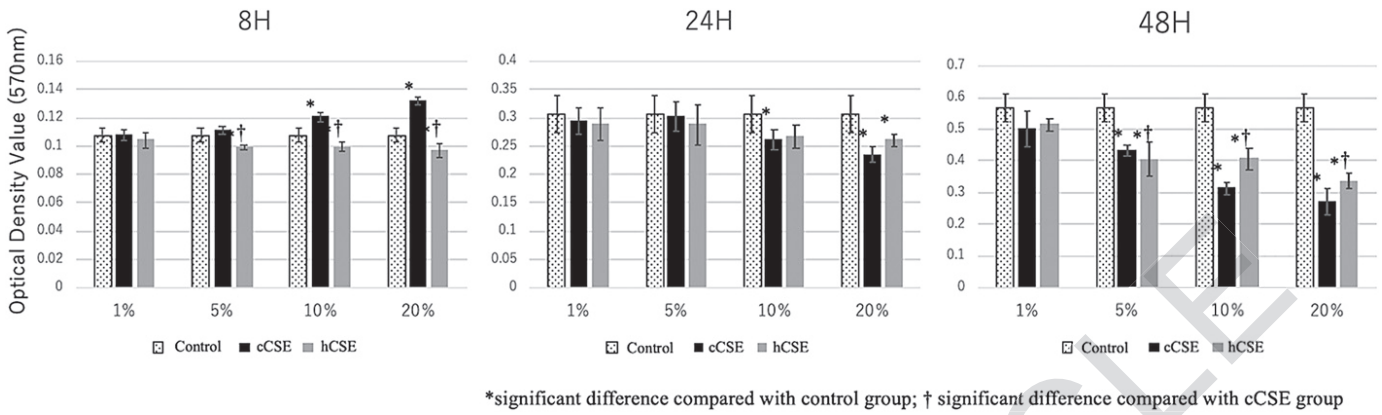

Fig. 3

Viability of MC3T3-E1 cells treated with the indicated concentrations of cigarette smoke extract. The Y axis shows the optical density value at 570 nm. The values are shown as the mean and standard deviation.

cCSE and hCSE Impairment of MC3T3-E1 Cell Viability

MC3T3-E1 viability was determined after incubation for 8, 24, and 48 hours with concentrated cCSE or hCSE (n = 6 for each group, concentration, and time point). Viability was reduced in a time-dependent manner. The viability of MC3T3-E1 cells incubated with 5% to 20% cCSE or hCSE for 48 hours was significantly lower than that of controls ($p < 0.05$ for all comparisons). Furthermore, the viability of MC3T3-E1 cells incubated with 10% and 20% cCSE for 48 hours was significantly lower than that of cells treated with 10% and 20% hCSE, respectively (Fig. 3). The viability of cells treated with 1% hCSE and 1% cCSE did not significantly differ from that of controls at any time point. Therefore, a 1% concentration of cCSE and hCSE was used to study osteoblastic differentiation to avoid the occurrence of indirect effects of treatment.

Osteoblastic Differentiation After Exposure to cCSE and hCSE

After a 2-week incubation period, ALP activity was significantly higher in cells incubated with BMP-2 than in cells incubated without BMP-2 (Fig. 4). In terms of the effect of cCSE and hCSE, ALP activity was significantly lower in cells treated with cCSE than in control and hCSE-treated cells. Levels in control and hCSE-treated cells also differed significantly (mean and SD, 452.4 ± 48.8 [untreated], 326.2 ± 26.2 [cCSE-treated], and

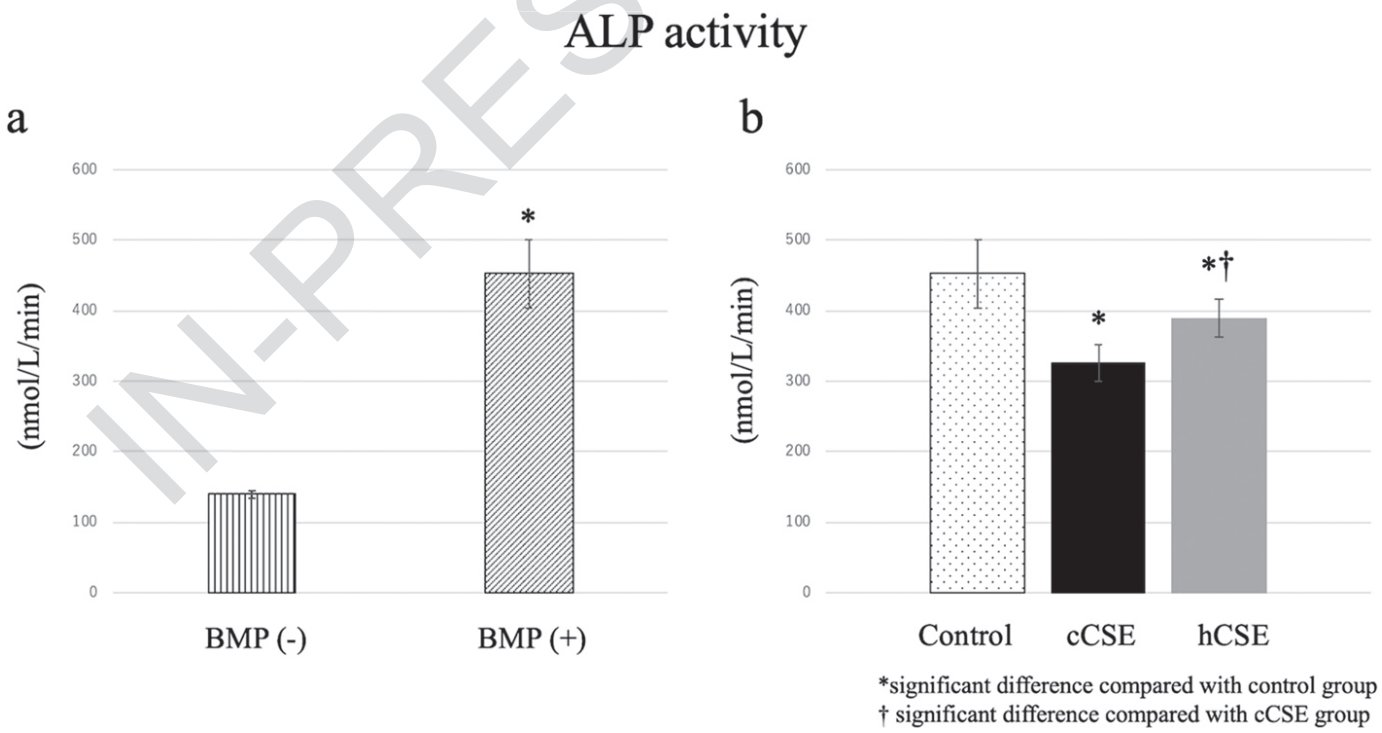

Fig. 4

Alkaline phosphatase (ALP) activity of **(a)** MC3T3-E1 cells incubated with and without BMP and **(b)** cells treated without CSE (control), with cCSE, and with hCSE. The values are shown as the mean and standard deviation.

389.9 ± 26.6 [hCSE-treated] mol/L/min; p = 0.002) (Fig. 4). The percentage of the area positive for Alizarin Red S was significantly lower in both the cCSE and hCSE groups (Fig. 5).

μCT and Biomechanical Analysis of the Femur

At the cortical region of the femoral midshaft, cCSE administration significantly decreased bone mineral density and bone mineral content 4 weeks after surgery compared with control femora. hCSE administration also decreased bone mineral content compared with controls, and cCSE treatment significantly increased cross-sectional area compared with controls. There was no significant difference in cortical thickness among the 3 groups (Table I). Figure 6 shows representative 3D-reconstructed μCT images of femora. cCSE and hCSE-exposed femora contained significantly lower quantities of new bone than control femora (Fig. 7). Moreover, compared with those in the controls, maximum load and elastic modulus were significantly decreased in both the cCSE and hCSE groups (Table I).

Discussion

As HTP use rapidly spreads[5-9], it is important to study its impact on bone-healing. In the current study, we found that HTP likely impairs cell viability and osteoblastic differentiation similar to combustible cigarettes. Furthermore, HTPs decreased bone mineral content and new bone volume and weakened the biomechanical properties of the femoral shaft in a rat osteotomy model.

We are aware of only a few independent experimental studies assessing the effects of HTPs. According to manufacturer data, HTPs contain reduced quantities of tobacco compared with conventional cigarettes[36]. The tobacco industry also claims that during HTP use, toxic chemical emissions are reduced because of the relatively lower device operating temperature[12-14]. Although manufacturer-sponsored studies have mostly shown the health benefits of switching from conventional cigarettes to HTPs, independent studies have indicated some potentially harmful consequences of HTP aerosol exposure[37]. The results of independent studies have suggested that toxic compounds are not completely removed from HTP aerosols and that the products are not risk-free[4,38]. The independent studies by Farsalinos et al. and Bekki et al. showed that heated tobacco sticks contain 70% to 80% of the nicotine concentration found in conventional cigarettes[39,40]. In the current independent study, high concentrations of hCSE impaired MC3T3-E1 cell viability, although the impairment was less profound than that in cells treated with cCSE for 48 hours. These results indicate that HTPs have toxic effects but that the effects could be relatively milder than those of combustible cigarettes.

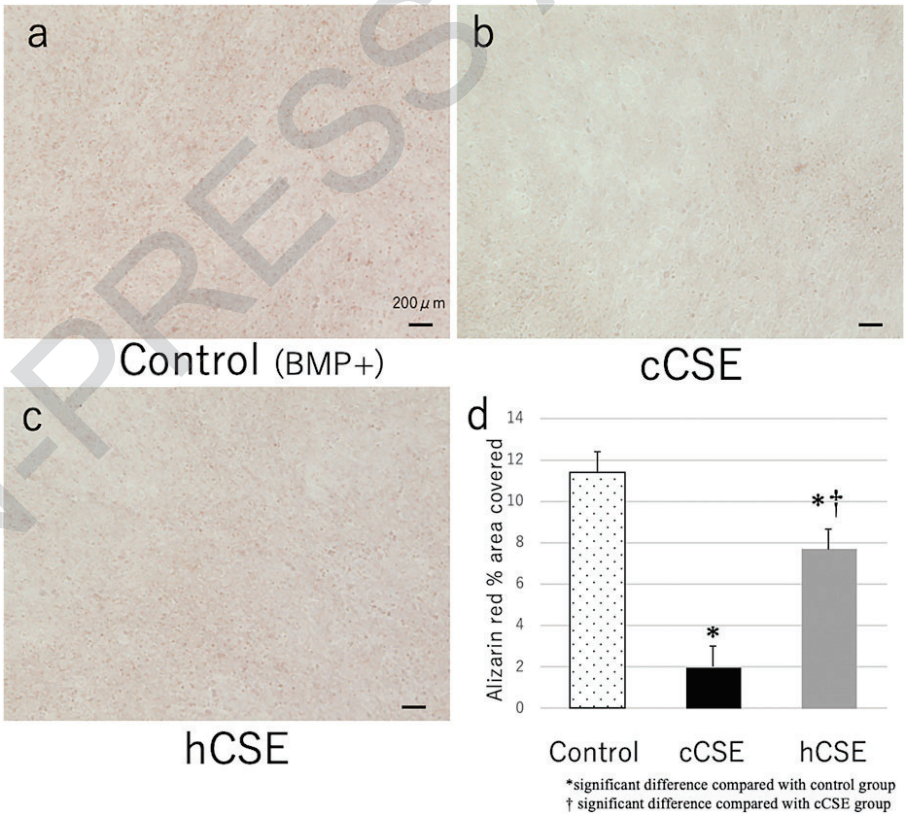

Fig. 5

Panels a, b, and c: Representative Alizarin Red S-stained samples of MC3T3-E1 cells treated **(a)** without CSE (control), **(b)** with cCSE, and **(c)** with hCSE. Staining indicates the extent of matrix mineralization. Alizarin Red S-positive mineralization and calcium content were altered in cCSE-treated and hCSE-treated cells as compared with controls. **Panel d:** Quantitative analysis of the percentage of the area positive for Alizarin Red S.

TABLE I μCT Analysis and Biomechanical Assessment of the Cortical Bone Region at the Femoral Midshaft*

	Control	cCSE	hCSE	P Value
μCT assessment				
Bone mineral content *(mg/cm³)*	107.0 ± 11.9	94.5 ± 13.0†	89.0 ± 10.1†	0.049
Bone mineral density *(mg)*	624.5 ± 38.5	537.8 ± 35.7†	577.4 ± 73.3	0.036
Cortical thickness *(mm)*	0.52 ± 0.004	0.54 ± 0.003	0.54 ± 0.003	0.559
Cross-sectional area *(mm²)*	7.76 ± 1.52	9.89 ± 0.85†	8.76 ± 1.33	0.033
Biomechanical assessment				
Maximum load *(N)*	71.4 ± 8.7	39.3 ± 13.9†	41.0 ± 13.5†	0.015
Elastic modulus *(N/m²)*	31.6 ± 12.3	15.2 ± 5.6†	10.3 ± 6.3†	0.019

*The values are given as the mean and the SD. †Significant difference compared with control group.

The present study used CSE for the experiments. Cigarette exposure via CSE allows researchers to use the same solution for in vitro and in vivo studies, which ensures that cells and animals are exposed to the same molecules. Additionally, it was possible to dilute the CSE to different concentrations. We successfully generated CSE from HTPs and normalized the concentrations by repeatedly confirming the optical density.

Although the effect of HTPs has not been established, the effects of combustible cigarettes and e-cigarettes on osteoblastic differentiation have been previously reported. Exposure to cCSE promotes the creation of a pro-osteoclastogenic environment, increasing the receptor activator of nuclear factor-kappa B (NF-κB) ligand-osteoprotegerin ratio and decreasing osteogenic activity[31]. In addition, systemic exposure to cCSE strongly increased serum pro-inflammatory and anti-inflammatory cytokine levels and promoted changes in bone microarchitecture in a dose-dependent fashion[31]. The results of the current study suggest that hCSE also has an inhibitory effect on osteoblastic differentiation in MC3T3-E1 cells by decreasing ALP activity and mineralization.

In terms of the relationship between the combustible cigarettes and bone union, Cyprus et al. used μCT scanning to examine the femora of mice that received cCSE injections for 25 days[31]. The authors reported that cCSE exposure decreased bone area and volume and that levels of pro-inflammatory cytokines in the blood increased. Chang et al. investigated the effect of cigarette smoking on fracture-healing with use of a femoral osteotomy model and performed experiments with use of a smoking chamber system[41]. The study revealed that vascular endothelial growth factor expression decreased and that bone-healing was delayed in the smoking group. The study by Sasaki et al. indicated that long-term cigarette smoke exposure impairs bone growth while increasing bone volume[42]. The findings

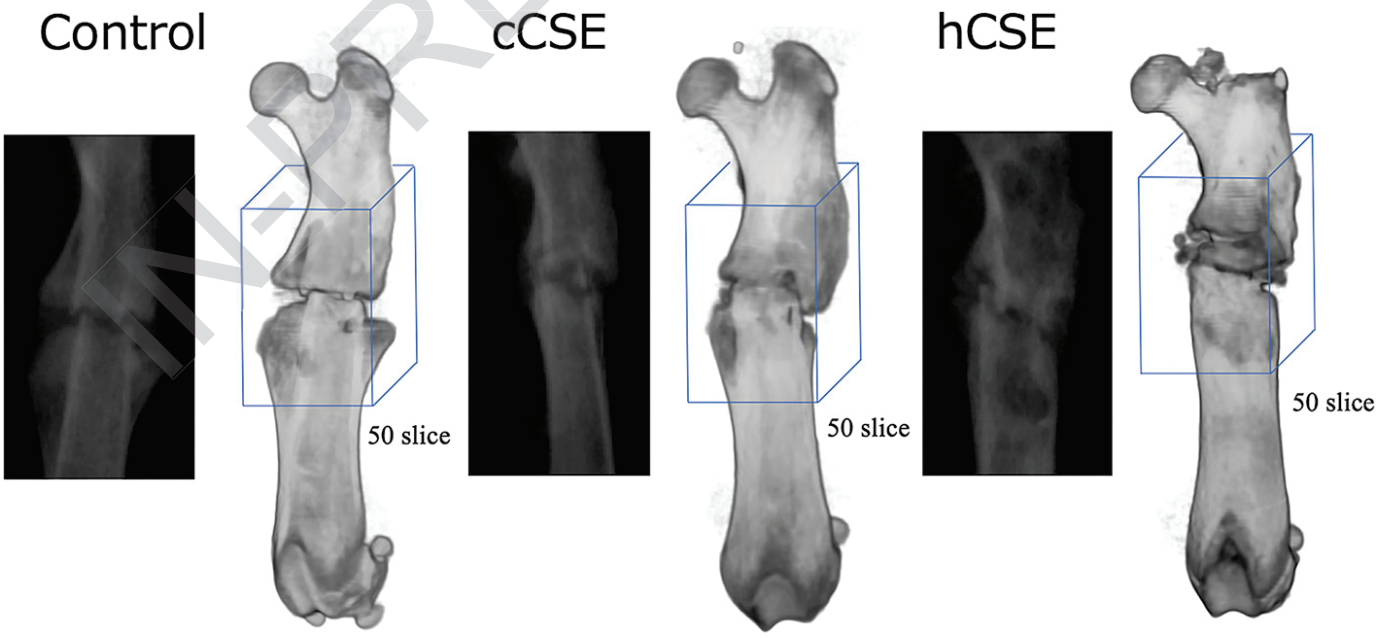

Fig. 6

Representative 3D-reconstructed μCT images (right image of each pair) and scout views (left image of each pair) of femora at 4 weeks after osteotomy.

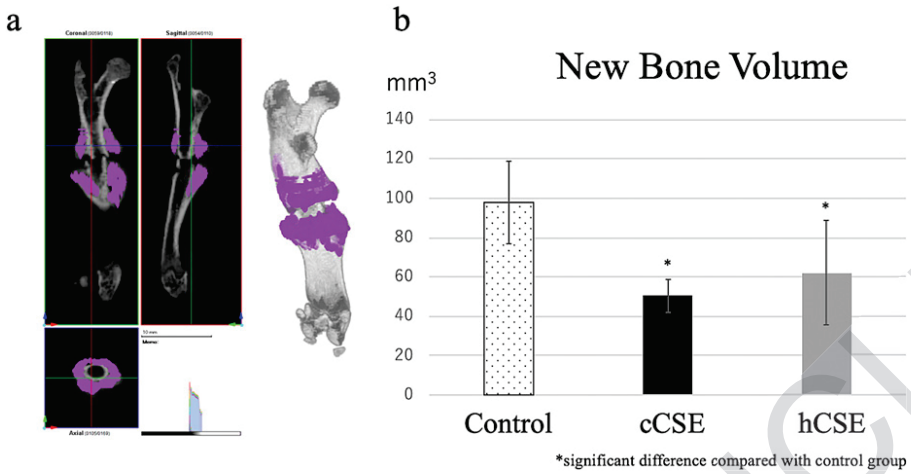

Fig. 7

Panel a: New bone volume was calculated with use of 3D image processing software (ExFact VR; Nihon Virtual Science). **Panel b:** Bar graph illustrating the quantification of new bone volume in the femoral midshaft.

of the present study revealed that both combustible cigarettes and HTPs significantly decrease bone mineral content and new bone volume after femoral osteotomy. In addition, the present study confirmed that HTPs can cause biomechanical deterioration of bones. Previous studies have demonstrated the biomechanical fragility of bones exposed to cigarette smoke[41,43]. Similarly, the present study also revealed that, like combustible cigarettes, HTP exposure led to weakened biomechanical properties.

The present study has some clinical implications. While HTPs are commonly understood to contain fewer types and reduced concentrations of toxicants that have been linked to diseases in combustible cigarette smokers, the present study adds to the growing body of evidence that suggests that a cautionary approach to HTP use is necessary. The findings of the present study indicate that HTPs have a negative impact on bone fracture-healing comparable with combustible cigarettes. When an orthopaedic surgery requires bone union, surgeons should recommend cessation of smoking conventional cigarettes and HTPs.

The present study had some limitations. First, because many substances in cigarettes have a negative effect on bone-healing, no single substance could be identified in our study. Second, serum nicotine levels were not assessed in this study. The effect of nicotine on fracture repair is well established[44]. Although the effects also could be due to multiple other substances, measurement of serum nicotine levels could be helpful to further characterize the potential differences. Third, an immortal cell line was used in these studies; although MC3T3 cells have been extensively used in previous studies on the effects of smoke on osteoblastic differentiation, primary cells may be a better model to investigate these aspects[45,46]. To overcome these limitations, further detailed study will be needed to assess the underlying mechanisms. Nevertheless, this independent study confirmed the negative impact of HTPs both in vivo and in vitro, and these results may contribute to the development of improved treatment strategies by orthopaedic physicians. In the future, an adequate period of cessation conventional combustible cigarettes and HTPs should also be established for further clinical application.

Conclusions

The use of HTPs impairs cell viability, osteoblastic differentiation, and bone fracture-healing similar to the use of combustible cigarettes. Orthopaedists should be aware of the negative impact of not only combustible cigarettes but also HTPs on bone-healing. ■

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