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

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Kazuya Nishino, Koji Tamai, Kumi Orita, Yusuke Hashimoto, Hiroaki Nakamura

概要	研究グループは、近年国内でも爆発的に普及している加熱式タバコが燃焼式タバコと同様に細胞生存率を低下させ、骨分化、骨癒合の阻害をもたらすことを明らかにしました。 燃焼式タバコの骨癒合への悪影響については広く知られていますが、加熱式タバコは体へ及ぼす害が少ないと一部では信じられています。そこで、本研究グループは加熱式タバコが骨癒合に与える影響を基礎的研究にて明らかにすることを目的に、燃焼式、加熱式でそれぞれタバコ抽出液を作成して前骨芽細胞に投与し、細胞生存率と骨分化誘導条件下における阻害作用を評価しました。また、ラットの大腿骨骨切りモデルに対しても各々の抽出液を投与し、4 週間後に骨組成、仮骨体積と力学強度を測定しました。その結果、細胞生存率は両群共に時間、濃度に依存して有意に低下しました。骨分化モデルでは、両群ともに ALP 活性が対照群より有意に低く、動物実験では、骨塩量、仮骨体積、また力学試験における最大荷重、弾性率が両群共に対照の抽出液を投与しない群より有意に低下しました。 本研究結果から、骨折治療中や骨癒合を必要とする手術に際しては燃焼式タバコだけではなく加熱式タバコの禁煙も勧めるべきであると考えられます。 《補足説明》 燃焼式タバコ：いわゆる一般的なタバコ。タバコ葉を燃焼させた排煙を吸入する。 加熱式タバコ：電子タバコとも称される。タバコ葉を加熱することで得られるエアロゾルを吸入する。燃焼式タバコより安全と広告された影響もあり、近年日本国内でも爆発的に使用者が増えている。 ALP（アルカリフォスファターゼ）活性：アルカリフォスファターゼは骨新生により骨芽細胞より放出されるため、骨形成マーカとして用いられる。 加熱式タバコ（電子タバコ）「も」骨折治癒に悪影響 ～骨折治療中は加熱式タバコでも禁煙を！～". 大阪市立大学. https://www.osaka-cu.ac.jp/ja/news/2021/210823. (参照　2021-08-23)

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Abstract

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. This 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 (MC3T3E1) 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 in vivo effects of CSEs, femoral midshaft osteotomy was performed in a rat model; thereafter, saline, cCSE, or hCSE was injected intraperitoneally, and bone union was assessed based on μCT and biomechanical analysis four weeks later.

Results

MC3T3E1 cell viability was reduced when treated with either cCSE or hCSE in a time- and concentration-dependent manner. ALP activity after osteoblastic differentiation of

cCSE-treated cells was significantly lower than that of both untreated and hCSE-treated cells **(control:452.4 ± 48.8, cCSE:326.2 ± 26.2, hCSE:389.9 ± 26.6mol/L/min, P=0.002)**. Moreover, levels of osteoblastic differentiation in untreated and hCSE-treated cells differed significantly. In vivo assessment of the femoral midshaft cortical region revealed that both cCSE and hCSE administration significantly decreased bone mineral content four weeks post-surgery compared to levels observed in untreated animals **(control:107.0 ± 11.9, cCSE:94.5 ± 13.0, hCSE:89.0 ± 10.1mg/cm^3, P=0.049)**. Additionally, cCSE- and hCSE-exposed femurs had significantly lower bone volumes than unexposed femurs. Biomechanical analyses showed that both cCSE and hCSE administration significantly decreased femoral maximum load and elastic modulus.

Conclusions

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

Clinical Relevance

HTP use negatively affects bone fracture healing to a degree similar to that of combustible cigarettes. Orthopedic surgeons should recommend HTP smoking cessation to improve bone union.

Introduction

A wide variety of tobacco products are available worldwide such as combustible cigarettes, electrical cigarettes, and heated tobacco products (HTPs). 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]. Since 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 (240–350°C) than conventional cigarettes (> 600°C), and produce lower levels of harmful chemicals such as tar than conventional cigarettes when used[4]. HTPs 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 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-D 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]. Further, there have been no studies that have assessed the effect of HTPs on osteoblastic differentiation and fracture healing. An independent investigation of effects of HTPs, which 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, osteoblast differentiation *in vitro*, and bone fracture healing *in vivo*. Further, HTP findings were compared with those of conventional combustible cigarettes using a cigarette smoke extract (CSE) method that allows researchers to standardize treatments.

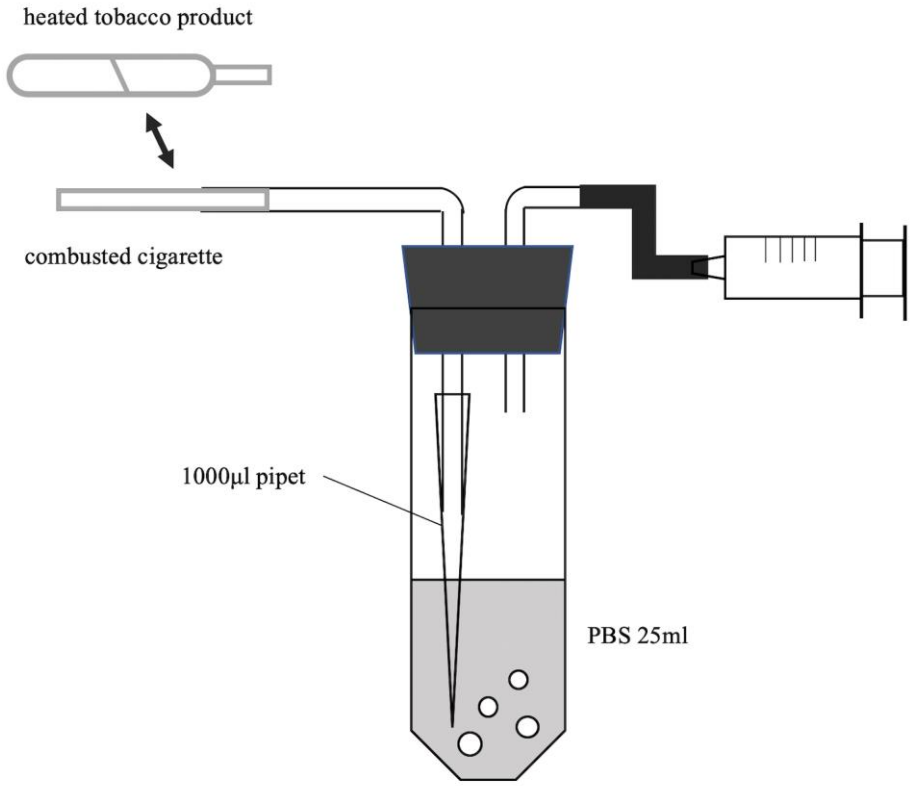
Materials and Methods

CSE Preparation

CSEs from combustible cigarettes (cCSE) and HTPs (hCSE) were generated as previously described (Figure 1)[24-27]. Briefly, combustible cigarette particulate matter was generated from commercially available cigarettes (Marlboro, Philip Morris, USA) with 12 mg tar and 0.9 mg nicotine per cigarette, using 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, and were subjected to negative pressure using a plastic syringe. The resulting extract was filter sterilized (0.22 μm Sterile Millex-GS filter units, Merck Millipore, Japan) before being used in the experiments. HTP particulate matter was obtained from a regular Marlboro Heat stick (Marlboro, Philip Morris, Japan). Smoke extract from HTP (hCSE) was generated using the same protocol that was used to create cCSE. The concentration of CSE generated from one, three, six, and nine cigarettes was determined and standardized by measuring the optical densities of materials at 320 nm (OD320) using a plate reader (Varioskan LUX, ThermoFisher, Japan). Each CSE concentration was determined thrice, and CSE from three cigarettes was defined as a 100% concentration of CSE[25,26]. The concentration of CSE from three cigarettes was measured at OD320 nm to confirm that it was within a standard deviation (SD) of its expected value before being used in an experiment. To fairly assess their harmful

effects, the numbers of cigarettes used to create cCSE and hCSE were matched, rather than optical density. Solutions were diluted (1, 5, 10, 20% of original concentrations) and immediately used for cell proliferation assays. An extract concentration that displayed no cytotoxicity was used to assess osteoblastic differentiation.

Figure 1



Cell Culture

The murine preosteoblast cell line MC3T3E was purchased from the RIKEN cell bank. For maintenance of stock plates, MC3T3E1 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

In Vitro Assays

The viability of the MC3T3E1 cells was assayed using an MTT Cell Proliferation/Viability Assay kit (R&D Systems, Inc., Minneapolis, MN, USA) 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 h so that wells contained 0% to 20% CSE. After 8, 24, and 48 h of incubation, an MTT assay was performed. Absorbance at 570 nm was determined using a microplate reader (U-3000 spectrophotometer, Hitachi, Japan). Six replicates for each group were assessed.

MC3T3E1 cells were plated on a 24-well plate (5×10^4 cells/well) and cultured with 300 ng/mL bone morphogenetic protein-2 (BMP2) for 2 weeks to induce osteoblastic differentiation[28,29]. Media were 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 MC3T3E1 cells were carried out to evaluate early- and late-phase osteoblastic differentiation[27,30]. ALP activity was assayed by measuring the OD405 nm using LabAssay ALP (Wako Pure Chemical Industries Ltd). 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 were quantified using Image J (National Institutes of Health) **by an independent observer**.

In Vivo Study

Animals and treatments

Wild-type male Lewis rats (Nihon SLC, Shizuoka, Japan) aged 8 weeks were used for *in vivo* experiments. All animals were maintained using a 24 h 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 (19042). Rats were anesthetized via subcutaneous injection of ketamine (50 mg/mL; Sankyo, Tokyo, Japan) and xylazine (0.2 mg/mL; Bayer HealthCare, Tokyo, Japan) 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 Kirschner wire (1.2 mm diameter) nailing. 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, cCSE,

or hCSE[31-33]. A 100% concentration of CSE was used. There were no exclusion cases.

μCT assessment

Rats were euthanized via CO_2 asphyxiation 28 days postoperatively. Femurs were collected and scanned with a μCT scanner (LaTheta LCT-100A scanner, Hitachi-Aloka Medical Ltd., Tokyo, Japan). 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 using LaTheta software (version 2.10, Hitachi-Aloka Medical Ltd., Tokyo, Japan). Parameters considered included bone mineral content (BMC) and bone mineral density (BMD). Geometric parameters, including cortical thickness (Ct.Th) and cross-sectional area (CSA), were also analyzed. The trabecular bone region was not analyzed due to 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 used to measure the volume of new bone using 3D image processing software (ExFact VR, Nihon Virtual Science, Inc. Japan).

Biomechanical assessment

Eighteen femurs at 4 weeks after surgery were used for biomechanical evaluation. A standardized three-point bending test was performed with each group using a bending tester

(EZ Graph; Shimadzu Corp., Kyoto, Japan)[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 mean ± SD. One-way analysis of variance with a post hoc t test was used to compare differences among the control, hCSE, and hCSE groups. Values of $P < 0.05$ were considered to indicate statistical significance. All analyses were performed using the R program (version 3.5.2; R Foundation for Statistical Computing, Vienna, Austria) and EZR computer software (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

Source of Funding

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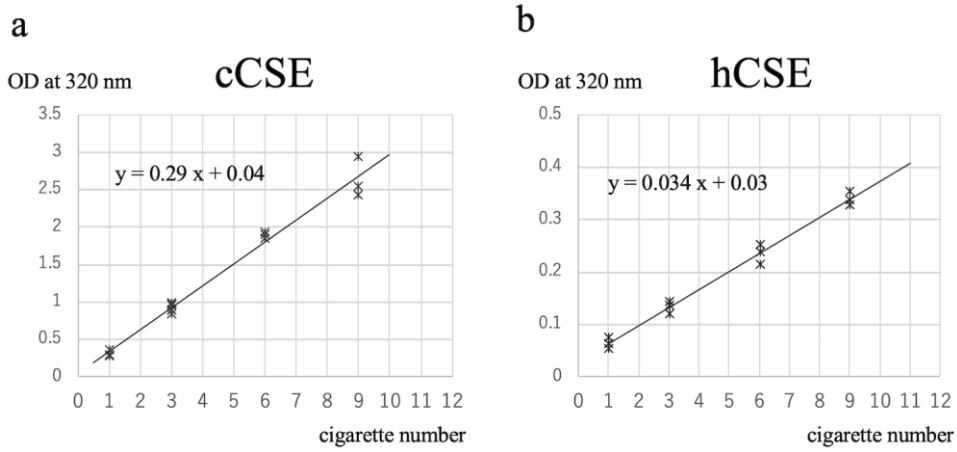
Results

Concentrations of cCSE and hCSE

Optical density increased in proportion with the number of cigarettes or HTPs added (Figure 2a,b). Using OD320 values, not only cCSE but also hCSE concentrations could be reproducibly quantified. Based on a previous report, the current study adopted the optical density of cCSE and hCSE generated from three cigarettes and sticks as 100% concentrations[25,26].

Figure 2

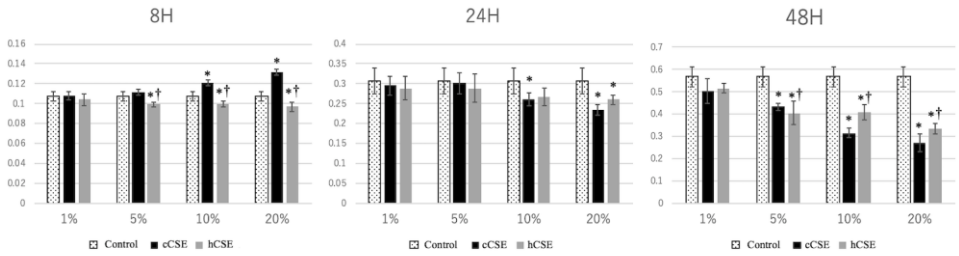


cCSE and hCSE Impair MC3T3E1 Cell Viability

MC3T3E1 viability was determined after incubation for 8, 24, and 48 h with concentrated cCSE or hCSE (n = 6 for each group, concentration, and time point). Viability was reduced in a time-dependent manner. After 48 h of incubation, MC3T3E1 cell viability after incubation with 5-20% cCSE or hCSE was significantly lower than that of controls ($p < 0.05$ for all comparisons). Further, the viability of MC3T3E1 cells incubated with 10% and 20% cCSE

for 48 h was significantly lower than that of cells treated with 10% and 20% hCSE, respectively (Figure 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.

Figure 3



Osteoblastic Differentiation After Exposure to cCSE and hCSE

After a 2-week incubation period, ALP activity was significantly higher in MC3T3E1 cells incubated with BMP-2 than in cells incubated without BMP-2 (Figure 4a). In terms of the effect of cCSE and hCSE, ALP activity was significantly lower in cells treated with cCSE than control and hCSE-treated cells. Levels in control and hCSE-treated cells also significantly differed (Figure 4b). The percentage of the area positive for alizarin red was significantly lower in both the cCSE and hCSE groups (Figure 5).

Figure 4

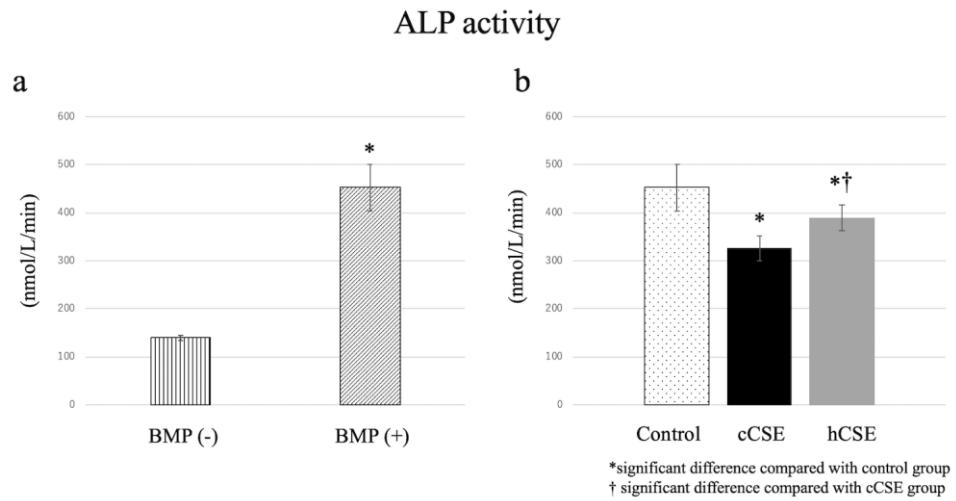
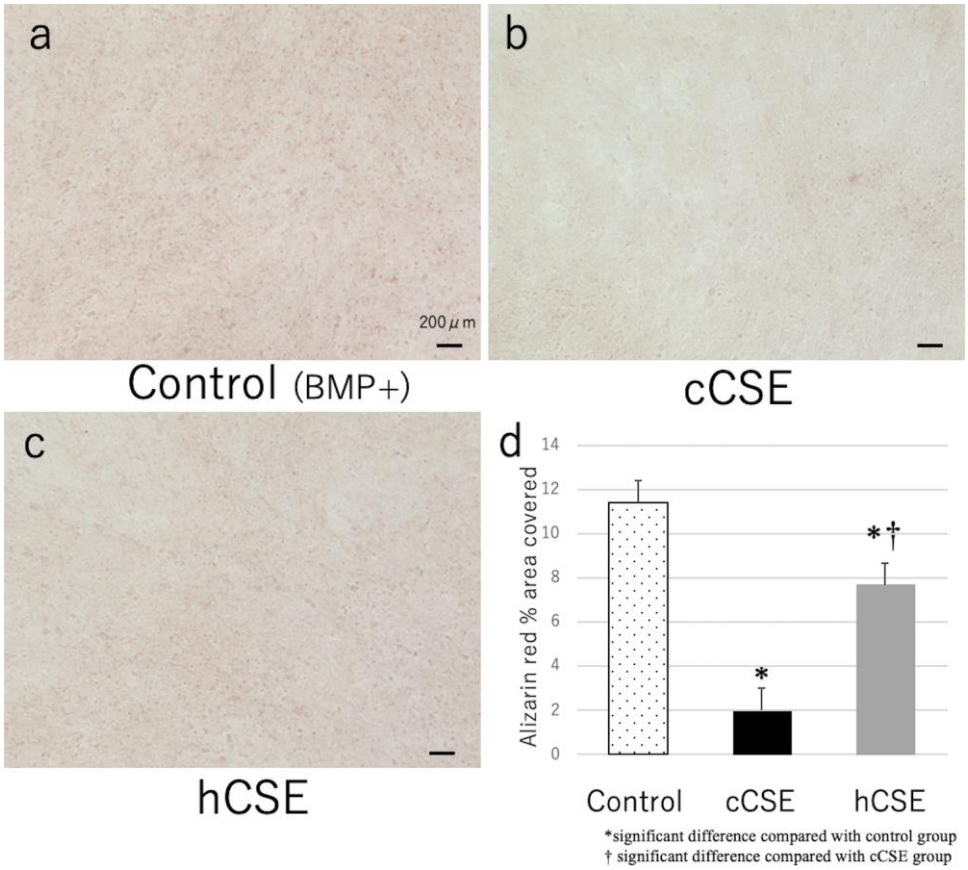


Figure 5



μCT and Biomechanical Analysis of the Femur

At the cortical region of the femoral midshaft, cCSE administration significantly decreased BMD and BMC 4 weeks post-surgery compared with control femurs. hCSE administration also decreased BMC compared with control, and cCSE treatment significantly increased CSA

compared with control. There was no significant difference in Ct.Th among the three groups (Table 1). Figure 6 shows representative, 3D-reconstructed μCT images of femurs. cCSE- and hCSE-exposed femurs contained significantly lower quantities of new bone than control femurs (Figure 7). Moreover, compared to those in the control groups, maximum load and elastic modulus were significantly decreased in both the cCSE and hCSE groups (Table 1).

Table 1 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.

Figure 6

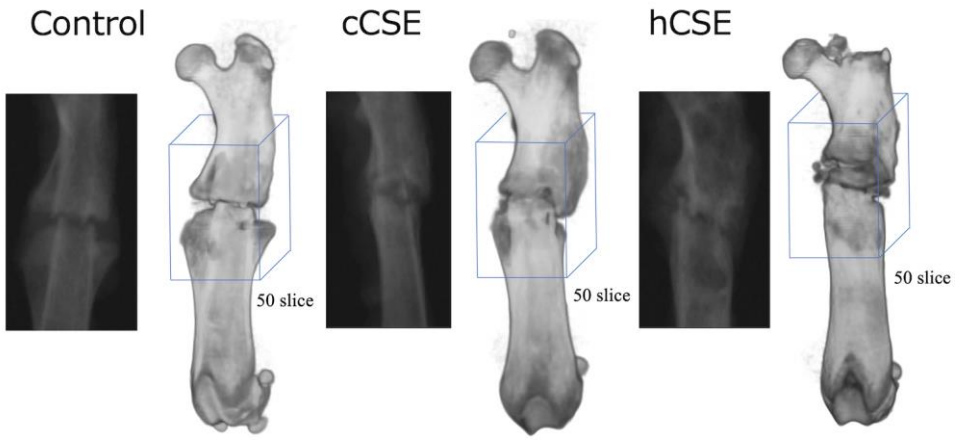
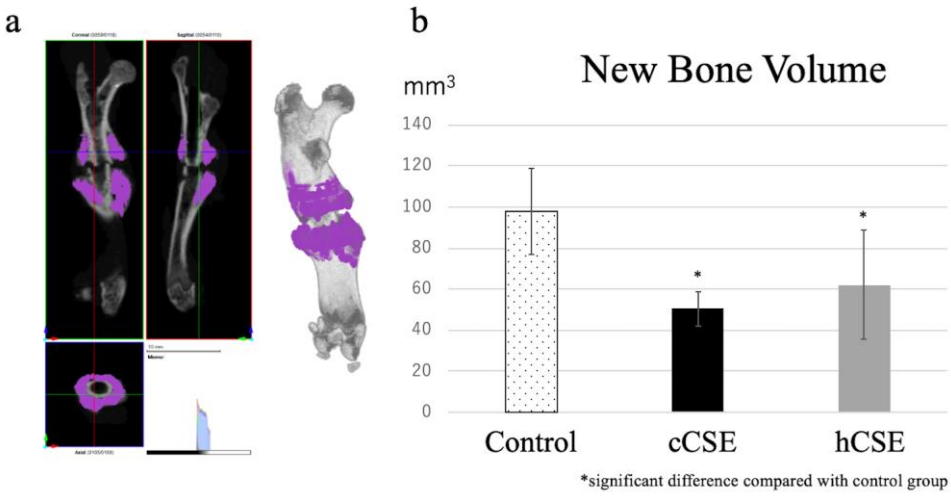


Figure 7



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 BMC and new bone volume, and weakened the biomechanical properties of the femoral shaft in a rat osteotomy model.

There are only a few independent experimental studies assessing the effects of HTPs. According to manufacturer data, HTPs contain reduced quantities of tobacco compared to conventional cigarettes[36]. The tobacco industry also claims that during HTP use, toxic chemical emissions are reduced due to 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 indicate some potentially harmful consequences of HTP aerosol exposure[37]. The results of independent studies suggest that

toxic compounds are not completely removed from HTP aerosols, and the products are not risk-free[4,38]. Other independent studies by Farsalinos et al. and Bekki et al. showed that heated tobacco sticks contain 70–80% of the nicotine concentration found in conventional cigarettes[39,40]. In the current independent study, high concentrations of hCSE impaired MC3T3E1 cell viability, though the impairment was less profound than that in cells treated with cCSE for 48 h. These results indicate that HTPs have toxic effects, but the effects could be relatively milder than those of combustible cigarettes.

This study used CSE throughout 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 electric cigarettes on osteoblastic differentiation have been previously reported. Exposure to cCSE promotes the creation of a pro-osteoclastogenic environment, increasing receptor activator of NF-κB ligand-osteoprotegerin ratio and decreasing osteogenic activity[31]. In addition, systemic exposure to cCSE strongly increased serum pro- and anti-inflammatory

cytokine levels and promoted changes in bone microarchitecture in a dose-dependent fashion[31]. The current results suggest that hCSE also has an inhibitory effect on osteoblastic differentiation in MC3T3E1 cells by decreasing ALP activity and mineralization.

In terms of relationship between the combustible cigarette and bone union, Cyprus et al. examined via μCT scan the femurs of mice that had been administered cCSE injections for 25 days[31]. They reported that cCSE exposure decreased bone area and volume and confirmed that levels of pro-inflammatory cytokines in the blood increased. Chang et al. investigated the effect of cigarette smoking on fracture healing using a femoral osteotomy model and performed experiments using a smoking chamber system[41]. The study revealed that vascular endothelial growth factor expression decreased, and delayed bone healing occurred in the smoking group. Another study indicated that long-term cigarette smoking exposure impairs bone growth while increasing bone volume[42]. Findings of this study revealed that both combustible cigarettes and HTPs significantly decrease BMC and new bone volume after femoral osteotomy. In addition, this 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, this study also revealed that, like combustible cigarettes, HTP exposure led to weakened biomechanical properties.

This 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, this study adds to the growing body of evidence that suggests that a cautionary approach to HTPs use is necessary. According to findings of this study, HTPs have a negative impact on bone fracture healing comparable to combustible cigarettes. When an orthopedic surgery requires bone union, surgeons should recommend smoking cessation of conventional cigarettes and HTPs.

There are some limitations in this study. 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 could also 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 orthopedic physicians. In the future, an adequate cessation period of conventional combustible cigarettes and HTPs should also be established for further clinical assistance.

Conclusions

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

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Figure legends

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

Figure 2. The relationship between the number of cigarettes and the optical density of cigarette smoke extract. Values for (a) combustible cigarettes and (b) heated tobacco products are shown.

Figure 3. Viability of MC3T3E1 cells treated with the indicated concentrations of cigarette smoke extract. **Y axis showed optical density value at 570 nm. The values are shown as the mean and standard deviation.**

Figure 4. Assessment of alkaline phosphatase (ALP) activity. ALP activity of MC3T3E1 cells incubated (a) with and without BMP and (b) **without CSE (control), with cCSE, and with hCSE** treatment are shown.

Figure 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.

Figure 6. Representative 3D reconstructed μCT images (right side of each column) and scout views (left side) of femurs 4 weeks post-osteotomy are shown.

Figure 7. An assessment of new bone volume is shown. (a) New bone volume is calculated using a 3D image processing software (ExFact VR, Nihon Virtual Science, Inc. Japan), and (b) the quantification of new bone volume in the femoral midshaft is shown.