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ORIGINAL ARTICLE

Green tea protects human osteoblasts from cigarette smoke-induced injury: possible clinical implication

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Abstract
Purpose Recent reports discuss the altered bone homeostasis in cigarette smokers, being a risk factor for osteoporosis and negatively influencing fracture healing. Cigarette smoke is known to induce oxidative stress in the body via an increased production of reactive oxygen species (ROS). These increases in ROS are thought to damage the bone-forming osteoblasts. Naturally occurring polyphenols contained in green tea extract (GTE), e.g., catechins, are known to have anti-oxidative properties. Therefore, the aim of this study was to investigate whether GTE and especially catechins protect primary human osteoblasts from cigarette smoke-induced damage and to identify the underlying mechanisms.
Methods Primary human osteoblasts were isolated from patients' femur heads. Cigarette smoke medium (CSM) was obtained using a gas-washing bottle and standardized by its optical density (OD_{320}) at λ=320 nm. ROS formation was measured using 2′7′dichlorofluorescein diacetate, and osteoblasts' viability was detected by resazurin conversion.
Results Co-, pre-, and post-incubation with GTE and catechins significantly reduced ROS formation and thus improved the viability of CSM-treated osteoblasts. Besides GTE's direct radical scavenging properties, pre-incubation with both GTE and catechins protected osteoblasts from CSM-induced damage. Inhibition of the anti-oxidative enzyme HO-1 significantly reduced the protective effect of GTE and catechins emphasizing the key role of this enzyme in GTE anti-oxidative effect.
Conclusions Our data suggest possible beneficial effects on bone homeostasis, fracture healing, and bone mineral density following a GTE-rich diet or supplementation.

Keywords Green tea extract · Catechins · Cigarette smoke medium · Oxidative stress · HO-1 · Primary human osteoblasts

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Introduction

Bone underlies a process of continuous remodeling during lifetime. Its homeostasis is highly complex and constitutes of four sequential phases. Activation precedes resorption, followed by reversal and formation [1]. Bone itself has three

different cell types with distinct functions. The first, osteoblasts, are responsible for bone's assembly and tissue generation. Later onwards, they support the bone structure itself as osteocytes, being the second cell type [2]. Osteoclasts constitute the third cell type responsible for resorption. A decrease in osteoblasts and their function can lead to several pathologies such as osteoporosis and delayed bone union. This in turn may contribute to an increased rate of pseudarthrosis in fracture healing.

Cigarette smoke is well established as a major health risk. Over 2.5% of its more than 6,000 molecular species are known toxic compounds. These compounds contribute to the pathogenesis of a variety of diseases, e.g., cancer, cardiovascular, and pulmonary diseases. However, in the past few years, more and more studies have demonstrated negative effects of cigarette smoke on bone [3–9]. Dependent on the concentration, nicotine has a biphasic impact on bone metabolism and osteoblast proliferation. High concentrations have been shown to inhibit proliferation whereas lower levels of nicotine stimulate proliferation [10]. Passive smoking generally has a negative influence on the bone mineral density, the bone volume, the trabecular structure, and the number of osteoblasts [11]. Furthermore, smoking is associated with delayed fracture healing, alterations in mineral content, and osteoporosis [7, 8, 12, 13]. Recent data suggest that toxins contained in cigarette smoke may not only initiate and exacerbate tissue injury but may also impair reparative processes via the initiation of inflammatory responses [14–16]. Hence, tissue destruction is exerted either through direct toxic effects (e.g., DNA damage), altered gene regulation, or indirectly through increased oxidative stress [8, 10, 17–20]. The increased oxidative stress may inhibit osteoblast differentiation [21]. On the contrary, it has been reported that reactive oxygen species (ROS) such as H_2O_2 or superoxide anion might not damage but stimulate osteoclast differentiation and thus favor bone resorption [22], which in combination with the expected reduced osteoblast function, leads to poor bone density. Moreover, in case of a fracture, the regeneration processes in smokers are impaired, which is often associated with prolonged hospital stays.

Thus, anti-oxidative dietary supplements, e.g., naturally occurring polyphenols, may become an important and moreover simple strategy to counter this process. Tea, as one of the most popular drinks in the world, contains high levels of polyphenols. Due to differences in the post-harvest treatment, green tea contains more polyphenols (mainly catechins) than black tea. Tea catechins particularly consist of epigallocatechin-3-gallat (EGCG), epigallocatechin, epicatechin-gallat, and epicatechin. The green tea extract (GTE) used for the present study (Sunphenon® 90LB, Taiyo Kagaku, Japan) is obtained from the leaf of traceable green tea (Camelliasinensis) and consist of >80% polyphenols, of which >80% are catechins, >40% EGCG, and <1% caffeine. A fundamental property of these molecules is their antioxidant capacity. Besides their function to directly scavenge ROS like the superoxide anion, oxygen singlet, and lipidic peroxyradicals, they can stabilize free ROS by means of hydrogenation or formation of complexes with oxidating species [23–25]. Based on that, polyphenols have been shown to have numerous biochemical and physiological benefits, including antioxidant, anti-microbial, anti-inflammatory, and cytoprotective activities [23, 26–28]. Clinical observations suggest that consuming green tea increases bone density and therefore reduces the risk of osteoporotic fractures [29, 30]. The underlying mechanisms, however, are not yet identified.

Thus, the aim of this project was to prove the protective effect of GTE and its catechins in primary human osteoblasts exposed to cigarette smoke medium (CSM). Respectively, we attempted to identify anti-oxidative mechanism of GTE. Just recently, we could show that various flavonoids are able to protect liver cells from etahnol-induced oxidative stress by interacting with the endogenous anti-oxidative defense system [31–33]. In this system, the heme-oxygenase-1 (HO-1) is of particular interest, as it is finely upregulated under "oxidative stress" and helps to protect the liver against damage from several chemical compounds such as acetaminophen, carbon tetrachloride, and heavy metals [34]. As there are several reports suggesting a major role of HO-1 during fracture repair [35, 36], we wanted to focus on this anti-oxidative enzyme in our study.

Materials and methods

Dulbeccos's phosphate buffered saline (DPBS), cell culture medium, and supplements are from PAA Laboratories, Cölbe, Germany; green tea extract from Sunphenon® 90LB, Taiyo Kagaku, Japan; and chemicals, from Sigma, Munich, Germany, if not stated differently.

Isolation and culture of primary human osteoblasts

Primary human osteoblasts were isolated from femoral heads of patient's undergoing total hip replacement. The bone was shredded into small pieces and thoroughly washed with DPBS. Afterward, the pieces were incubated with an equal volume of digestion buffer (DPBS, 0.07% collagenase II; Biochrom AG, Berlin, Germany) for 1 h at 37°C. The supernatant was centrifuged in order to remove the collagenase. The obtained cells were cultivated with osteogenic medium (MEM/Ham's F12, 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM L-ascorbate-2-phosphate, and 50 μM β-glycerol-phosphate) [37]. The medium was changed twice a week

[38] while cells were expanded. Experiments were performed in passage 3 with a homogenous cell population, being negative for CD14 and CD45 and positive for CD90 and CD105 (flow cytometry).

Generation of cigarette smoke medium (CSM)

CSM was prepared freshly before each experiment, as suggested by the International Organization for Standardization (ISO 10362-2) and the Federal Trade Commission [39]. Briefly, filters were removed from the cigarettes (Marlboro, Philip Morris, Munich, Germany), which were placed on a standard gas washing bottle (Lenz Laborglas GmbH & Co. KG, Wertheim, Germany) connected to a peristaltic pump producing negative pressure [40, 41]. Three cigarettes were blown through 35 ml medium for 15 min with a rate of 1 toke (2 s)per min. Before using the freshly prepared CSM, it was filtered (0.22 μm filter, Sarstedt, Nürnberg, Germany) and its optical density at 320 nm (OD_{320}) was determined using a plate reader (BMG Labtech, Offenburg, Germany) [42].

ROS measurement

Prior to experiments, osteoblasts were incubated with 10 μM 2′,7′-dichlorfluorescein-diacetate in serum-free culture medium for 30 min at 37°C [43]. After washing the cells twice with DPBS, osteoblasts were stimulated with CSM. After 15 min, the formed fluorescence, representing ROS levels, was measured using a plate reader (fluorescence, ex/em=485/520 nm).

Viability measurement

Viability was measured by resazurin conversion. Briefly, the cells were incubated for 1 h at 37°C with 1/10 volume of a 0.025% (*w/v*) resazurin solution (in DPBS). Resazurin is converted to fluorescent resorufin via the reduction reactions of metabolically active cells. Fluorescence was measured (ex/em=540/590 nm) with a plate reader. Viability is given as percent of control (untreated cells).

Statistics

Data sets are shown as mean±SEM of at least three independent experiments ($N \geq 3$) measured as triplicates ($n=3$). Results were compared by one-way analysis of variance followed by Bonferroni's multiple comparison test (GraphPad Prism Software, El Camino Real, USA). A $p<0.05$ was taken as the minimum level of significance.

Results

Cigarette smoke reduces viability of osteoblasts in a concentration- and time-dependent manner

The viability of primary human osteoblasts decreased after incubation with CSM time- and dose-dependently. With 24 h stimulation, even with the highest dilution (OD_{320}= 0.2) of CSM, 100% toxicity was obtained. After 12 h, CSM with an $OD_{320} \geq 0.2$ showed 100% toxicity (Fig. 1a). Therefore, experiments were performed with only a 4-h incubation interval. At this time-point, plotting the viability against the optical density of the CSM showed a close correlation between both parameters. Approximately 50% viability remained after stimulation with CSM with an $OD_{320}=0.75$ for 4 h (Fig. 1b). Thus, for the following experiments, we decided to use a CSM with an OD_{320}= 0.8 and an incubation time of 4 h to standardize the setup. The experiments were divided into three settings: (1) pre-incubation setting with 4 h incubation with sub-toxic concentrations of GTE or catechins followed by 4 h exposure to CSM; (2) co-incubation setting with 4 h simultaneous exposure to sub-toxic concentrations of GTE or catechins and CSM; (3) post-incubation setting with 4 h exposure to CSM followed by 4 h incubation with sub-toxic concentrations of GTE or catechins.

Determination of sub-toxic concentrations of GTE and catechins

First, we determined the concentration range in which GTE and catechins are non-toxic. After 4 h treatment with 0, 50, 100, and 200 μg/ml, GTE the viability of osteoblasts was not affected (Fig. 1c). Similarly, after 4 h treatment with 0, 50, 100, and 200 μM catechins, the viability of osteoblasts was not affected (Fig. 1d). Thus, these concentrations were used for further experiments.

Primary human osteoblasts show increased ROS formation after incubation with CSM

ROS were detectable after only 5 min incubation with CSM (data not shown). After 15 min, the accumulation of ROS was significant and remained for approximately 2–3 h. A significant increase in ROS formation was only observed when cells were incubated with CSM with an $OD_{320} \geq 0.5$ ($p<0.01$/Fig. 2a).

GTE and catechins reduce ROS formation in CSM-treated osteoblasts in a dose-dependent manner

ROS formation was measured in osteoblasts pre-incubated for 4 h with 0, 50, 100, and 200 μg/ml GTE or 0, 50, 100,

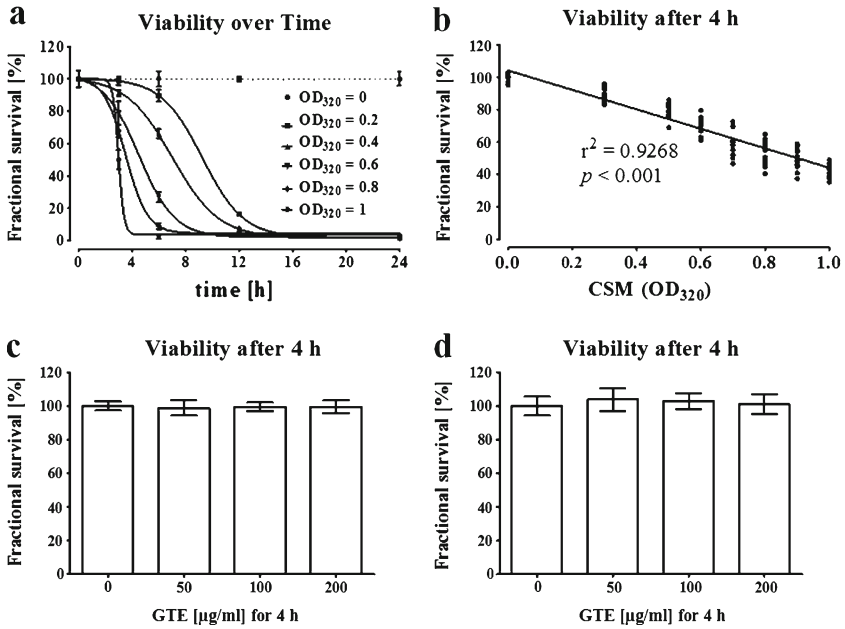

Fig. 1 CSM damages primary human osteoblasts in a time- and concentration-dependent manner. **a** Primary human osteoblasts (N=3, n=4) were treated with different concentrations (OD_{320}=0, 0.2, 0.4, 0.6, 0.8, and 1) of CSM for 0, 3, 6, 12, and 24 h, after stimulation viability was determined by resazurin conversion. **b** Primary human osteoblasts (N=5, n=4) were treated with different concentrations (OD_{320}=0, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, and 1) of CSM, after 4 h viability was determined by resazurin conversion. Plotting the viability against the densities of the CSM showed a positive correlation between both parameters. **c** Primary human osteoblasts (N=3, n=4) were treated with different concentrations of GTE (0, 50, 100, and 200 µg/ml) or **d** catechins (0, 50, 100, 200 µM), after 4 h viability was determined by resazurin conversion. None of the tested concentrations affected viability of the osteoblasts

and 200 µM catechins followed by a 15-min exposure to CSM (OD_{320}=0.8) as well as after 15 min co-incubation of the substances. In both settings, CSM significantly induced ROS formation. Both pre- and co-incubation with all three concentrations of GTE and catechins significantly reduced ROS formation dose-dependently (Fig. 2b–e).

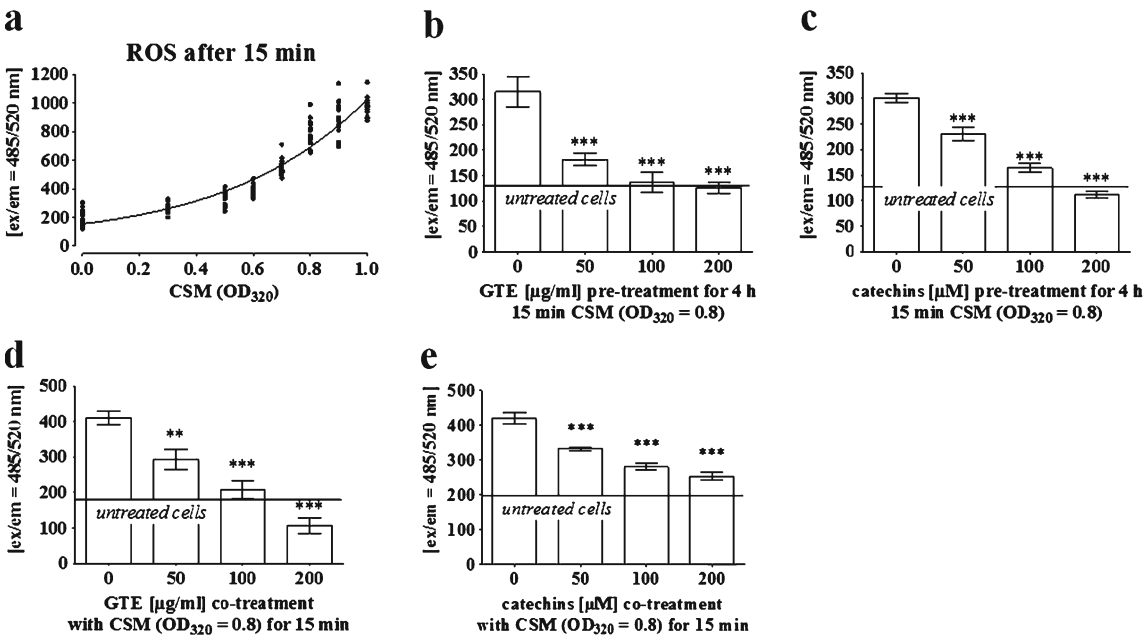

Fig. 2 GTE and its major components catechins reduce CSM-induced ROS production in primary human osteoblasts. **a** After 15 min, CSM induced the production of ROS in primary human osteoblasts (N=3, n=4). The induction remained stable for 2–3 h (data not shown). Pre-incubation of primary human osteoblasts (N=3, n=4) with sub-toxic concentrations of **b** GTE (0, 50, 100, and 200 µg/ml) or **c** catechins (0, 50, 100, and 200 µM) significantly reduced the formation of ROS in CSM (OD_{320}=0.8)-exposed primary human osteoblasts. Similar results were observed during co-incubation with CSM and **d** GTE or **e** catechins. $^{**}p<0.01$; $^{***}p<0.001$ as compared with CSM-treated cells

GTE and catechins protect primary human osteoblasts from CSM-induced damage

Consequently, pre- and co-incubation with GTE or catechins improves the viability of CSM-treated osteoblasts in a dose-dependent manner (Fig. 3a–d). Most interestingly, the post-incubation setting with 4 h exposure to CSM followed by 4 h incubation with GTE or catechins also reduced CSM-dependent damage in primary human osteoblasts, reaching levels up to 90% of untreated cells (Fig. 3e–f).

Inhibition of HO-1 with zinc protoporphyrine (ZnPP9) reverses the protective effect of GTE and catechins in primary human osteoblasts

We determined the $LD_{50/24\ h}$ of the HO-1 inhibitor zinc protoporphyrine (ZnPP9) to be 12.3±1.1 μM (Fig. 4a). As proof whether the protective effect of GTE and catechins is dependent on HO-1 expression, we repeated the viability measurement of CSM-treated cells after a pre- and co-incubation with 200 μg/ml GTE or 200 μM catechins in the presence or absence of a non-toxic dose (10 μM) of ZnPP9. It became evident that the presence of ZnPP9 significantly diminished the protective effect of GTE and catechins (Fig. 4b–c).

Discussion

We could clearly demonstrate CSM's toxic effects on primary human osteoblasts. Cell damage is time- and concentration-dependent. Our data show that CSM induces osteoblast damage alongside with an increase in ROS formation that was already seen after a 15 min treatment with

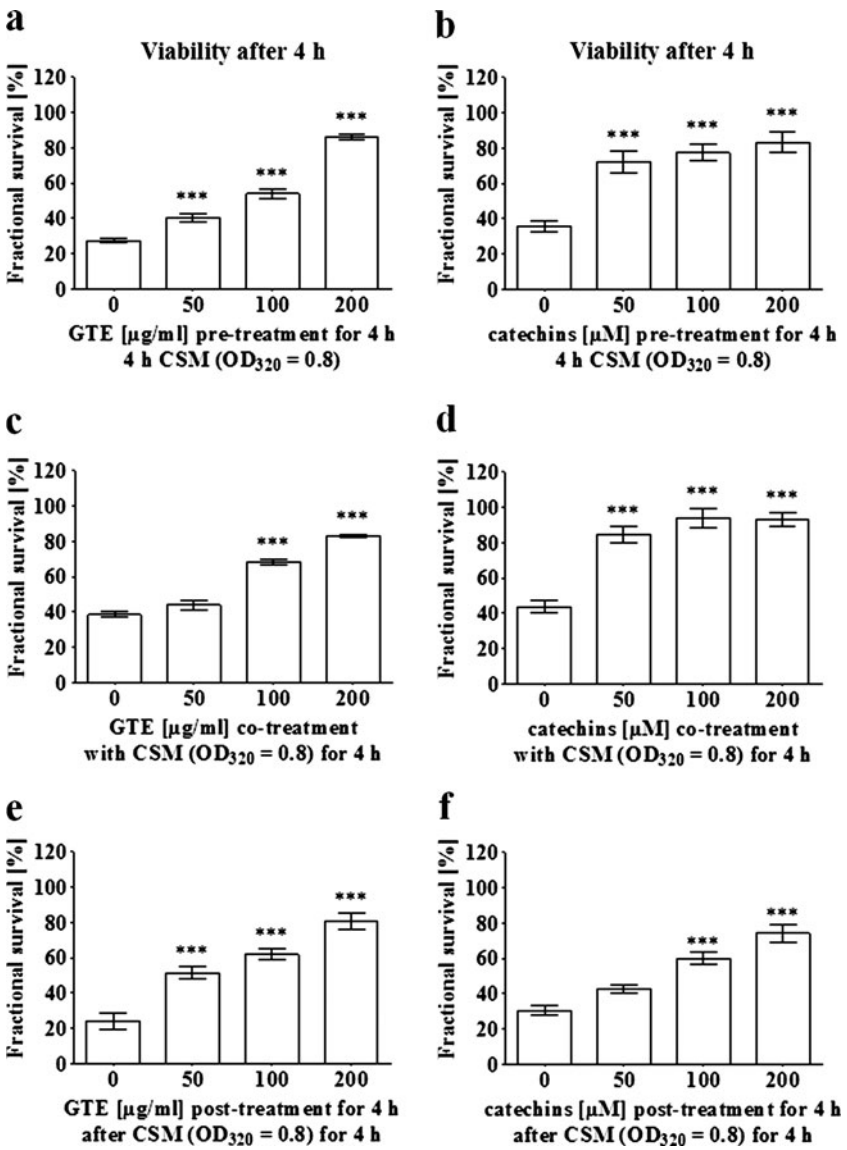

Fig. 3 GTE and catechins dose-dependently reduce CSM-induced cellular damage in human osteoblasts. Pre-incubation of primary human osteoblasts (N=3, n=4) with sub-toxic concentrations of **a** GTE (0, 50, 100, and 200 μg/ml) or **b** catechins (0, 50, 100, and 200 μM) significantly increased viability after CSM (OD_{320}=0.8) exposure. Similar results were observed during co-incubation with CSM and **c** GTE or **d** catechins. Interestingly, even post-incubation of CSM-damaged cells with **e** GTE or **f** catechins improved their viability. ***p<0.001 as compared with CSM-treated cells

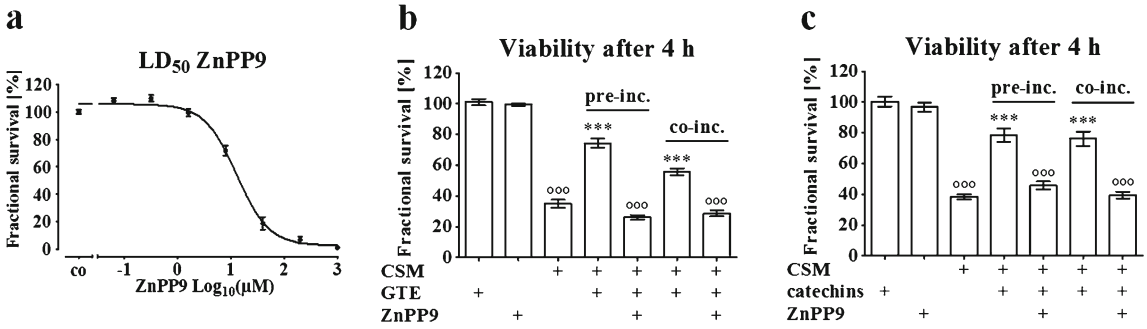

Fig. 4 Protective effect of GTE and catechins is dependent on HO-1 activity. **a** Primary human osteoblasts (N=3, n=4) were treated with different concentrations of the HO-1 inhibitor ZnPP9 (0, 0.064, 0.32, 1.6, 8, 40, 200, and 1,000 μM), after 24 h viability was determined by resazurin conversion. The HO-1 inhibitor ZnPP9 (10 μM) reduced the protective effect of **b** GTE (200 μg/ml; pre- and co-incubation) and **c** catechins (200 μM; pre- and co-incubation) on primary human osteoblasts (N=3, n=4) treated with CSM (OD_{320}=0.8) for 4 h. Viability was determined by resazurin conversion. °°°p<0.001 as compared with untreated cells, ***p<0.001 as compared with CSM-treated cells

CSM. This is supported by several publications, suggesting a positive correlation between increased oxidative stress and cell death in osteoblasts, influencing fracture healing, bone regeneration, and bone mineral density [3, 4, 6, 7, 13, 44, 45]. This emphasizes the need for new treatment strategies to reduce oxidative stress in patients, e.g., smokers that are reported to have strongly elevated oxidative stress levels. GTE and its major components, catechins, have been attributed anti-oxidative properties in the past [27]. This is supported by the report of Wu et al. describing GTE's potential to improve bone mineral density in habitual tea drinkers [30]. This is astonishing, as one would expect a poor bioactivity of substances in tea due to the processing with boiling water and the difficult uptake in the intestines. Furthermore, the underlying molecular mechanism of which GTE exerts its protective effect is poorly understood. For example, Rothem et al. ascribe cigarette smoke positive proliferative effects in osteoblasts concentration-dependently [10]. This is contradictory to our findings that clearly show a positive correlation between increasing ROS levels and cell damage in primary human osteoblasts, suggesting bone loss in vivo. This is supported by several publications, investigating the effect of cigarette smoke on bone mineral density and fracture healing in rodents [3, 4, 6, 7, 13, 44, 45] that clearly show a negative effect of cigarette smoking on bone. Our results suggest a direct toxic effect of cigarette smoke on the bone-forming osteoblasts. Furthermore, Bai and co-workers could prove that ROS, e.g., H_2O_2 or superoxide anions, are involved in the oxidative stress-related bone loss by stimulating osteoclast differentiation and thus bone resorption [22]. This suggests that the observed reduction in bone mineral density observed in smokers is both actively by an increased bone resorption and passively by a reduced bone formation, both being related to increased oxidative stress levels. An increase in ROS might furthermore affect the cells' viability by damaging their DNA. Additionally, poor cell viability might be caused by a dys-balance between ROS and nitric oxide, including *S*-nitrosothiols, nitric oxide synthase activities, or a reduction of glutathione, as observed in cigarette smoke-treated alveolar epithelial cells [46].

A way to treat these alterations in patients is reducing the oxidative stress level. Several in vivo studies about green tea consumption and the rate of osteoporosis in patients exist [47–51]. Hence, we attempted to analyze the different protective potentials of GTE. GTE contains polyphenols, e.g., catechins, in high concentrations and is by far more potent than an oral green tea uptake. Our experimental setup showed positive effects of GTE and catechins not only on primary human osteoblasts co-incubated with CSM, but also when cells were pre-incubated with GTE. These benefits could possibly be used as prophylaxis in smokers known to be at high risk for osteoporosis and other bone homeostasis disorders. We propose that this prophylactive effect may be due to an increased expression of the anti-oxidative enzyme HO-1, as the addition of the HO-1 inhibitor ZnPP9 effectively blocked the protective effects of both GTE and catechins on CSM-exposed osteoblasts. This is supported by the finding of Chae and co-workers that show that HO-1 is necessary to protect ostoeblasts from tumor necrosis factor-alpha-induced apoptosis [52]. Although the work from Lin and colleagues suggests an inhibitory effect of HO-1 on osteoblast maturation and mineralization [53], Bargallo and co-workers were able to show improvement of osteogenic stem cell differentiation by HO-1 [35]. Interestingly, in our experiments, GTE and catechins even proofed to be effective in the post-incubation setting. This possibility to halt and reverse the CSM-dependent cell damage opens up new therapeutic opportunities. Patients with increased oxidative stress levels suffering from chronic diseases, osteoporosis, and delayed fracture healing might benefit from GTE

and catechin supplementation. Thus, our data indicate a great potential of GTE and its major components catechins to deliver new therapeutic possibilities. They could be administered to treat patients with elevated oxidative stress levels, especially as high ROS levels have been attributed a vast amount of different pathologies such as bone loss-related diseases [54].

Conclusion

Our findings demonstrate that green tea extract may influence bone mineral density in smokers by reducing their oxidative stress level, enhancing their osteoblast viability, and in doing so, supporting bone healing in general. Dietary supplementation with GTE and/or catechins appears to be an effective and simple way to counter high ROS levels—not only associated to the bone. Preventive administration before surgery, supportive consumption, as well as the administration of GTE and/or catechins on already sick persons may positively influence the bone cell viability and reduce the oxidative stress.

Conflicts of interest None.

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