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 Regenerative Medicine

Impact of resveratrol on bone repair in rats exposed to cigarette smoke inhalation: histomorphometric and bone-related gene expression analysis

F. C. Franck¹, B. B. Benatti²,
 D. C. Andia¹, F. R. Cirano¹,
 R. C. Casarin¹, M. G. Corrêa¹,
 F. V. Ribeiro¹

¹Dental Research Division, School of Dentistry, Paulista University, São Paulo, SP, Brazil; ²School of Dentistry, Federal University of Maranhão, São Luís, MA, Brazil

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Abstract. This study investigated the effect of resveratrol on bone healing and its influence on the gene expression of bone-related markers in rats exposed to cigarette smoke. Two calvarial defects were created in each of 60 rats, which were assigned equally ($n = 20$) to three groups: (1) resveratrol (10 mg/kg) + smoke exposure (SMK + RESV); (2) placebo + smoke exposure (SMK + PLA); or (3) placebo + no smoke exposure (NS + PLA). Substances were administered daily for 30 days following surgery. Smoke inhalation was started 7 days before surgery and continued for 30 days after surgery. One defect was processed for histomorphometric analysis and the other was used for mRNA quantification of bone-related gene expression by qPCR. The remaining defect was smaller in the SMK + RESV (2.27 ± 0.61 mm, $P = 0.0003$) and NS + PLA (2.17 ± 0.74 mm, $P = 0.0005$) groups than in the SMK + PLA group (3.12 ± 0.47 mm). Higher levels of Runx2 were observed in the NS + PLA group than in the smoke exposure groups (vs. SMK + PLA, $P = 0.002$; vs. SMK + RESV, $P = 0.052$); levels of Lrp-5 were also higher in the no smoke exposure group (vs. SMK + RESV, $P = 0.009$; vs. SMK + PLA, $P = 0.003$). Resveratrol therapy decreased RANKL/OPG expression when compared to placebo (SMK + RESV vs. SMK + PLA, $P = 0.017$). Dkk1 levels were decreased in the SMK + RESV group when compared to the SMK + PLA ($P = 0.006$) and NS + PLA groups ($P = 0.005$). In conclusion, resveratrol optimizes the repair of critical-sized bone defects, up-regulating the gene expression of important bone remodelling markers in rats exposed to cigarette smoke inhalation.

Key words: resveratrol; plants; medicinal; wound healing; rats; gene expression; smoking.

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It is well established that tobacco use has a negative effect on bone healing, as supported by innumerable experimental and clinical studies showing the harmful impact of smoking on the repair of bone defects, on fracture union, on bone regeneration, and on the healing process of the tooth extraction socket¹⁻³. In addition, cigarette smoking has been related to an augmented prevalence and severity of peri-implant lesions and is a risk factor for bone loss around implants^{4,5}.

The negative impact of cigarette smoking on bone healing is related to some pathophysiological mechanisms that predispose smokers to bone loss, including alterations in the receptor activator of nuclear factor kappa B (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) system (RANK–RANKL–OPG system), bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF- β), and alkaline phosphatase (ALP), all key bone-related factors^{1,6-8} that are also involved in bone angiogenesis⁶. In line with this, Yamano et al. showed that the expression levels of osteopontin (OPN), type II collagen (COL-II), BMP-2, bone sialoprotein (BSP), and core-binding factor alpha-1 (Cbfa1) were down-regulated in rats at 4 weeks after exposure to nicotine, compared to the non-nicotine exposure group⁹. Moreover, cigarette smoke contains various chemical toxins that induce reactive oxygen species (ROS) and cause cellular oxidative stress, which can affect the expression of inflammatory signalling molecules and the differentiation of osteoblasts and osteoclasts^{10,11}.

Different therapies aimed at minimizing the deleterious effects of tobacco use on bone health, through the stimulation of local osteogenesis and the microcirculation, and/or via modulation of the host immunoinflammatory response, have been investigated. In this context, Franco et al. evaluated the regeneration of bone defects implanted with biomaterial and stimulated with low-level laser therapy in rats exposed to cigarette smoke¹². They found that the osteogenic effect of the laser therapy protocol was not sufficient to improve bone regeneration in the presence of smoking. With regard to alternative therapies, the impact of natural products used to counter the effects of tobacco smoke on bone repair has been investigated in other studies¹³.

In recent years, one of the most studied products prepared from plants has been the compound resveratrol (3,5,4'-trihydroxy-trans-stilbene), a polyphenol found mainly in the skin of dark-coloured grapes and

in red wine. It has been shown by the scientific community that resveratrol presents robust anti-oxidant, anti-tumoural, and anti-inflammatory effects¹⁴, and has beneficial effects in the prevention and treatment of some diseases^{15,16}. Additionally, resveratrol has favourable effects on inflammatory markers and oxidative stress in smokers, reducing the concentrations of C-reactive protein and triglycerides, and increasing the levels of total anti-oxidants, again suggesting its anti-inflammatory and anti-oxidant therapeutic roles in these patients¹⁷. Furthermore, resveratrol seems to present the capacity to inhibit the deleterious effects of cigarettes on different tissues^{18,19}, including bone tissue²⁰.

With regard to bone metabolism, Uysal et al. demonstrated that the local application of resveratrol had positive effects on bone neof ormation by stimulating bone repair in the intermaxillary suture and reducing the time required for postoperative retention in rats submitted to surgical maxillary expansion²¹. Additionally, a previous study involving some of the present authors revealed that the use of resveratrol favoured the biomechanical retention of implants and the bone repair of critical-sized calvarial defects in rats²². Interestingly, resveratrol has been shown to stimulate the formation of bone and to inhibit bone loss processes, increasing the expression of runt-related transcription factor 2 (Runx2) and OPN, both of which have osteogenic properties²³. Indeed, it was demonstrated in the authors' previous study that there was higher gene expression of BMP-2, BMP-7, and OPN in repaired bone tissue of animals treated with resveratrol when compared to the placebo group²². Recent studies showing the benefits on bone metabolism of resveratrol have reported, among other mechanisms, an increase in the signalling of the canonical Wnt pathway; this stimulates mesenchymal cell nuclear stabilization of β -catenin in a dose- and time-dependent manner, in addition to promoting the activation of osteoblastic differentiation^{24,25}.

Given the potential of resveratrol to optimize bone repair and promote the inhibition of bone loss, and considering the damage caused by smoking during the osseous repair processes, it would be relevant to investigate the possible benefits of this natural compound in stimulating bone repair in the presence of tobacco use. While there are some molecular data showing the positive effects of resveratrol on the immunoinflammatory cascade and processes related to bone metabolism, the influence of this compound on the repair

of bone defects in the presence of smoking is not yet known. Research into the host response using animal models for bone repair is critically important to better understand the host immunoinflammatory response and to develop improved treatments in circumstances of smoking. This study aimed to determine the influence of this natural compound on the bone formation processes in the presence of cigarette smoking and to establish at least some of the biological mechanisms.

Materials and methods

Animals

Sixty 10-week-old male Wistar rats were included in the animal cohort; they had a mean weight of 334 ± 32 g at the beginning of the study. The rats were acclimatized for 15 days before use, and were kept in temperature-controlled cages, exposed to a 24-h light–dark cycle (12:12 h), with free access to water and food (Labina; Purina, Paulinia, SP, Brazil) in the animal facility of the university. The experimental procedure was approved by the animal care and use committee of the university.

Treatment groups

The animals were allocated to three groups: (1) SMK + RESV: daily administration of resveratrol (10 mg/kg) and smoke exposure ($n = 20$); (2) SMK + PLA: daily administration of placebo and smoke exposure ($n = 20$); and NS + PLA: daily administration of placebo and no smoke exposure ($n = 20$).

A stock solution of resveratrol (R5010-500MG, molecular weight 228.2; Sigma-Aldrich Ltd, São Paulo, SP, Brazil) was prepared in Tween-80 (P4780; Sigma-Aldrich, São Paulo, São Paulo, Brazil) and further diluted in water to working concentrations. The placebo solution was composed of the same quantities of Tween-80 and water as used in the preparation of resveratrol. The therapies were administered via gavage for 30 days following surgery²² (Fig. 1).

The rats were submitted to daily inhalation (three times per day) of cigarette smoke using a method involving an acrylic chamber ($45 \times 25 \times 20$ cm), in which five rats at a time were exposed to the smoke of 10 cigarettes (concentration 1.3 mg of nicotine, 16.5 mg of tar, and 15.2 mg of carbon monoxide). The chamber comprises two interconnected acrylic boxes. Lighted cigarettes are placed in the first box. This box has an external pump connected to it through which air is pumped to

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