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Research Paper
Bone Healing

Resveratrol improves bone repair by modulation of bone morphogenetic proteins and osteopontin gene expression in rats

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Abstract. This study investigated the effect of resveratrol on bone healing and its influence on the gene expression of osteogenic markers. Two calvarial defects were created and one screw-shaped titanium implant was inserted in the tibia of rats that were assigned to daily administration of placebo (control group, $n = 15$) or 10 mg/kg of resveratrol (RESV group, $n = 15$) for 30 days. The animals were then sacrificed. One of the calvarial defects was processed for histomorphometric analysis and the tissue relative to the other was collected for mRNA quantification of bone morphogenetic protein (BMP)-2, BMP-7, osteopontin (OPN), bone sialoprotein (BSP), osteoprotegrin (OPG), and receptor activator of NF-κB ligand (RANKL). Implants were removed by applying a counter-torque force. Histomorphometric analysis revealed higher remaining defect in the calvarial defects of the control group than the RESV group ($P = 0.026$). Resveratrol increased the counter-torque values of implant removal when compared to control therapy ($P = 0.031$). Gene expression analysis showed a higher expression of BMP-2 ($P = 0.011$), BMP-7 ($P = 0.049$), and OPN ($P = 0.002$) genes in the RESV group than in the control group. In conclusion, resveratrol improved the repair of critical-sized bone defects and the biomechanical retention of implants. Indeed, this natural agent may up-regulate the gene expression of important osteogenic markers.

Key words: Resveratrol; Plants; Medicinal; Wound healing; Rats; Osseointegration; Gene expression.

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The control of bone metabolism represents a key factor in obtaining a reduction in bone tissue resorption in inflammatory diseases and in predictable bone healing, and also in producing improvements in osseointegration, especially when conditions that reduce the effectiveness of bone healing are present.[1–3] Thus, local and systemic agents have been tested, although none has been described as a potential and safe mediator of bone metabolism.

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Although bisphosphonates are used to control bone resorption and increase bone deposition, their use has been associated with osteonecrosis of the jaws.[4] Parathyroid hormone PTH(1–34) intake increases bone healing, reduces bone resorption, and improves the osseointegration of implants.[5–8] However, it has adverse effects, such as hypercalcemia, associated with chronic use.[9] Thus, alternatives to these synthetic drugs that could promote similar benefits with reduced adverse effects have been the target of many studies, and the use of products and their components prepared from plants has increased.

Among these compounds, resveratrol (trans-3,5,4′-trihydroxystilbene), found in black grape skin,[10] has become important in light of various findings of pharmacological and clinical studies. Beyond the protective effect of resveratrol against several tumour and pathological mechanisms,[11–13] its inhibitory effect on osteoclast differentiation and its potential to induce bone formation have also been studied.[14–17] However, the effect of this substance on the repair of bone wounds has yet to be evaluated in detail. The purpose of this study was to evaluate the effects of resveratrol on the bone repair of calvarial defects and around titanium implants, and, in addition, to determine the impact of this agent on the gene expression of key modulators of physiological and pathological bone remodelling.

Materials and methods

Animals

The animal cohort comprised 10-week-old male Wistar rats ($n = 30$), weighing 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 of equal time, and had free access to water and food (Labina; Purina1, 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

Animals were allocated to one of two groups: a control group ($n = 15$), which received daily administration of a placebo solution for 30 days, and the RESV group ($n = 15$), which received daily administration of 10 mg/kg resveratrol for 30 days.[18] A stock solution of resveratrol (R5010-500MG; Sigma–Aldrich Ltda, São Paulo, SP, Brazil), molecular weight 228.2, was prepared in ethanol and further diluted in water to obtain working concentrations. The placebo solution was composed of the same quantities of ethanol and water as used in the preparation of resveratrol. The therapies were administered via gavage for 30 days following surgery.

Calvarial defects

General anaesthesia was obtained by intramuscular administration of ketamine hydrochloride (10 mg/kg) and xylazine hydrochloride (10 mg/kg). After cranial dorsal shaving, the surgical site was scrubbed with iodine, and a 15-mm midsagittal linear incision was made through the skin of the scalp. A full-thickness flap, including periosteum, was then reflected, exposing the calvarial bone. Two circular critical-sized defects of 5 mm in diameter[19] were made on each side of the parietal bone with the use of a trephine drill (AS Technology, São José dos Campos, SP, Brazil) under irrigation with sterile saline, preserving the medium sagittal bone (Fig. 1a, b). The soft tissues and periosteum were then repositioned for total coverage and sutured.

Implant placement

During the same surgical procedure, a screw-shaped titanium implant (AS Technology, São José dos Campos, SP, Brazil) was inserted in one tibia of each animal, according to a method described previously.[20] After shaving the tibia and cleaning the skin with iodine surgical soap, an incision of approximately 1.0 cm in length was made, and the bone surfaces of the tibia were surgically exposed by blunt dissection. Under saline irrigation, a bicortical implant bed was drilled at a rotary speed not exceeding 1500 rpm. A screw-shaped, commercially available pure titanium implant, 4.0 mm in length and 2.2 mm in diameter, was inserted until the screw thread was completely embedded in the bone cortex (Fig. 1c–e). Lastly, the soft tissues were replaced and sutured.

Postoperative period

The animals were evaluated daily throughout the experiment to check for possible clinical or toxicological symptoms. Thirty days after the start of the study, the animals were euthanized by CO_2 inhalation. The calvarial bones were removed. The right side of the calvarial bone (including the repair area) was fixed in 4% neutral formalin for histomorphometric analysis, and the surgical site of the left side was stored in RNAlater (Ambion Inc., Austin, TX, USA) for gene expression analysis. The tibia was dissected to expose the implants for torque force evaluation on implant removal.

Histomorphometric analysis

The specimens were demineralized in a solution containing equal parts of 50% formic acid and 20% sodium citrate (Chemco Indústria e Comércio Ltda, Campinas, SP, Brazil) for 40 days.

Paraffin semi-serial sections (6 μm) were obtained in an antero-posterior direction and stained with haematoxylin and eosin. Twenty sections were uniformly selected from the beginning to the end of the defect and analyzed with a light microscope (Zeiss, Jena, Germany) under 2.5× magnification. Using an image analysis system (Image-Pro; Media Cybernetics, Silver Spring, MD, USA), linear measurements of the healed and the remaining defect (i.e., the empty space between bone margins of the calvarial defects) were calculated from one side to the other side of the bone by the same examiner. All measurements were performed by the same calibrated blinded examiner, following intra-examiner calibration by evaluating seven non-study photomicrographs presenting calvarial defects. The examiner recorded the linear measurements of the remaining healing defect of all photomicrographs twice within 24 h. The intra-class correlation coefficient showed 97% reproducibility for this parameter.

Torque force evaluation on removal of the implants

Each tibia was dissected to expose the implant, allowing the attachment of a torque metre with a scale range of 0.1–10 N/cm and divisions of 0.05 N/cm (Mark-10, Long Island, NY, USA). A wrench was adapted to the implant head to apply torque in the reverse direction to implant placement until complete rupture of the bone–implant interface was signalled by rotation of the implant. The torque force value obtained in N/cm was considered as the torque necessary for the breakdown of osseointegration.[21,22]

Gene expression analysis

The calvarial sample stored in RNAlater at −70 °C was used for the evaluation of mRNA levels of bone morphogenetic protein (BMP)-2, BMP-7, osteopontin (OPN), bone sialoprotein (BSP), osteoprotegrin