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ORIGINAL ARTICLE

Nitric oxide metabolite levels during the ectopic osteoinduction in rats

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Abstract Nitric oxide (NO) is a cell-signaling molecule that has diverse biological functions. Recent evidence suggests that its production may regulate the metabolism of the osteoblasts and osteoclasts**.** The aim of this study was to evaluate levels of nitrite and nitrates (NO metabolites) during ectopic osteoinduction in rats. Eighteen male Sprague–Dawley rats (body weight 200–300 g) were used in this study. All animals were anesthetized and the right and left flank areas were shaved, and under aseptic conditions, a muscular pouch was created in each flank: the left was filled with 20 mg of demineralized bone matrix and the right remained empty (sham). Radiographs were taken at 2, 4, and 6 weeks after surgery to trace the ectopic bone formation and muscle mineralization. Blood samples were taken before (as baseline values) and at 2, 4, and 6 weeks after surgery. The mean values of NO metabolites after 6 weeks were significantly higher ($p<0.05$) than baseline data and at 2 weeks post-surgery. Results from this study indicate that the ectopic osteoinduction caused increased activity of the osteoblasts which subsequently caused increased serum levels of NO metabolites (nitrites and nitrates).

Keywords Nitric oxide · Ectopic osteoinduction · DBM · Rat

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Introduction

Bone is known to contain at least two types of cells, osteoblasts—the bone-forming cells—and osteoclasts—the bone-resorbing cells. Bone remodeling requires the proper maintenance and balance of these two types of cells, and imbalance often leads to pathophysiology. Bone metabolism is regulated by hormones and cytokines that affect the resorption and deposition of bone. These processes are normally coordinated so that deposition is balanced with resorption of bone (McClung 2003; Lindsay 2004). Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), function as potent local stimulatory signals for bone resorption. These factors interact with the receptors present on osteoblasts to modulate bone formation. Osteoclast resorption is modulated by the release of unknown signals collectively known as the osteoclast resorption-stimulating activity (Ueno et al. 1998).

Nitric oxide (NO) is a free-radical gas consisting of a very small lipophilic molecule which rapidly diffuses and reaches the cytoplasmic components resulting in the activation of diverse biological functions (Hamed et al. 2006). It has been implicated as an important gas mediator and secondary messenger molecule in biological processes involving vasodilatation, inflammation, and neurotransmission. Recent evidence suggests that its production may regulate the metabolism of osteoblasts and osteoclasts (Collin-Osdoby et al. 1995; Corbett et al. 1999; Diwan et al. 2000). NO is now known to be produced by various cells in different organs,

including smooth muscle cells, mesenchymal cells, neurons, platelets, hepatocytes, macrophages, fibroblasts, and epithelial cells. It also regulates smooth muscle cell tone, platelet aggregation, adhesion, cell growth, apoptosis, neurotransmission, and injuries as well as infection-induced immune reactions (Lincoln et al. 1997; Pacher et al. 2007). It is involved in a variety of conditions such as inflammation (Farrell et al. 1992; Stefanovic-Racic et al. 1994), arthritis (Farrell et al. 1992; Kaur and Halliwell 1994; Sakurai et al. 1995), osteoporosis (Wimalawansa et al. 1996), sepsis (Buttery et al. 1994), and aseptic loosening (Hukkanen et al. 1997).

NO is generated by the conversion of the amino acid L-arginine to L-citrulline in a reaction catalyzed by a family of isoenzymes arising from three distinct genes. Two of these isoenzymes, endothelial NO synthase (eNOS; type III) and neuronal NOS (nNOS; type I), are thought to be constitutive in nature. They are calcium-dependent and generally considered to be involved in physiological homeostasis generating basal levels of NO. The synthesis and activity of the third isoform, inducible NOS (iNOS; type II), is induced by many proinflammatory cytokines and are thought to be calcium-independent (Nathan and Xie 1994; Garcia and Stein 2006). NO has been shown to have significant effects on osteoblasts, osteoclasts, and osteocytes. Both eNOS and iNOS have an important role in the regulation of the proliferation and function of osteoblasts (Ralston 1994; Hukkanen 1995; Riancho et al. 1995).

Osteoinductive properties of allogenic and xenogenic demineralized bone matrix (DBM) has been studied previously (Solheim 1998; Torricelli et al. 1999; Bigham et al. 2008). Demineralized bone-induced heterotopic osteogenesis in rodents has been used as an experimental model to investigate osteoinduction itself as well as to study the effects of several influences on osteoinduction (Urist 1965; Reddi and Huggins 1972; Hagen et al. 1992). The aim of this study was to evaluate nitrite and nitrate (NO metabolites) levels during ectopic osteoinduction in rats.

Materials and methods

Animals

Eighteen male Sprague–Dawley rats (body weight 200–300 g) were used in this study. They were maintained in plastic cages in a room with a 12-h day/night cycle and an ambient temperature of 21°C, and were allowed ad libitum access to water and standard laboratory pellets. Animal selection, management, surgical protocol, and preparation followed the routines approved by the Institutional Animal Care and Use Committee, Shahrekord University, Shahrekord, Iran.

Preparation of demineralized bovine fetal bone matrix

A 6-month-old bovine fetus (Holstein) was collected from the local slaughterhouse. Metacarpal bones were dissected aseptically, and all soft tissue was removed. The retrieved bone was then sliced, and demineralization was performed as described by Reddi and Huggins (1972). The harvested bone was cleaned of soft tissue and marrow, washed in sterile distilled water with continuous stirring, then washed three times in 95% ethanol for 15 min, rinsed in ether for 15 min, and finally air-dried overnight. The cleaned and dried growth plates were then milled (Universal Mill A-20; Tekmer Co, Cincinnati, OH, USA) to obtain 400–700-μm granules and then demineralized in 0.6 NHC1 three times for 1 h (50 ml HC1/g of bone). The bone powder was rinsed with several changes of sterile distilled water to adjust the pH, three times in 95% ethanol and once in ether. The bone powder was air-dried and stored in sterile plastic containers at 4°C until required for implantation. This entire process was performed under sterile conditions (except for the milling), and a sample was cultured to demonstrate that specimens contained no bacterial or fungal contamination.

Surgical technique

All animals were anesthetized by means of a subcutaneous injection of ketamine 100 mg/kg and xylazine 30 mg/kg and the right and left flank areas were shaved and prepared aseptically with povidon iodine and draped with sterile drapes. Under aseptic conditions, a muscular pouch was created in each flank: the left was filled with 20 mg of DBM and the right remained empty (sham).

Postoperative evaluation

Radiological evaluation

Radiographs were taken at 2, 4, and 6 weeks after surgery to trace ectopic bone formation and muscle mineralization.

Assessment of nitrite and nitrate

Blood samples were taken before surgery (as base line values) and 2, 4, and 6 weeks after surgery. Serum samples were prepared from rats in each group and stored at −20°C. Measurement of nitrite and nitrate were based on the reduction of nitrate to nitrite by cadmium. The nitrite produced was determined by the Griess reaction. Using this method, the first serum sample was deproteinized by adding $ZnSO_4$ (75 mmol/L) and NaOH (55 mmol/L) solutions. After centrifuging, the supernatant was recovered and diluted with glycine buffer (45 g/L, pH 9.7). Cadmium granules (2–2.5 g) were rinsed three times with deionized

distilled water and swirled in a 5 mmol/L $CuSO_4$ solution in glycine–NaOH buffer (15 g/L, pH 9.7) for 5 min to activate them. Freshly activated cadmium granules were then added to the pretreated deproteinized serum. After continuous stirring for 10 min, the samples were transferred to appropriately labeled tubes for nitrite determination by the Griess reaction. Griess reagent 1 (1% sulfanilamide in 5% phosphoric acid) was added to the sample tubes and then incubated for 10 min at room temperature, protected from light. Finally, Griess reagent 2 was dispensed (0.1% *N*-napthylethylenediamine dihydrochloride in water) to all samples, and the absorbance was measured within 10 min in a spectrophotometer with a 540-nm filter (Navarro-Gonzalvez et al. 1998).

Statistical analysis

Data were analyzed by one-way analysis of variance and Duncan test as a post hoc using SPSS 15.0 software.

Results

All surgeries were carried out successfully without any anesthetic or postoperative complications. Radiological assessment showed abdominal muscle mineralization and ectopic new bone formation 6 weeks after the intramuscular xenogenic DBM implantation (see Fig. 1).

The mean values of NO metabolites after 6 weeks was significantly ($p<0.05$) higher than the baseline data and at 2 weeks post-surgery. There were no significant differences between the NO metabolite levels between the baseline data and either 2 or 4 weeks after surgery ($p>0.05$; see Fig. 2).

Fig. 1 Radiograph showing abdominal muscle mineralization and ectopic new bone formation at 6 weeks postoperatively (*white arrow*)

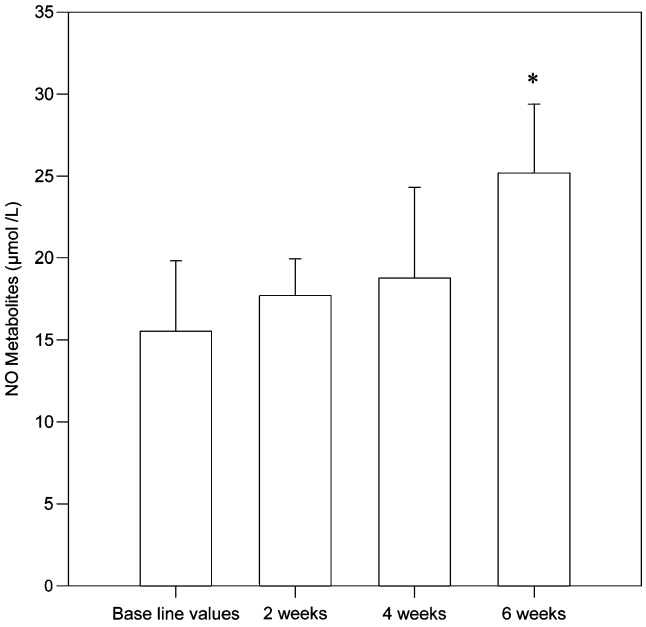

Fig. 2 Serum levels of NO metabolites (nitrites and nitrates) before and after surgery (*asterisk* NO metabolites after 6 weeks was significantly higher ($p<0.05$) than baseline and at 2 weeks post-surgery)

Discussion

The process of bone demineralization with hydrochloric acid decreases antigenic stimulation and may enhance the release of bone morphogenic protein (BMP; Riley et al. 1996). BMPs stimulate local undifferentiated mesenchymal cells to transform into osteoblasts (osteoinduction). These proteins promote the chondroblastic differentiation of mesenchymal cells followed by new bone synthesis by endochondral osteogenesis (Urist 1979, 1983). Our previous study proved that xenogenic DBM has potential osteoinductive properties orthotopically (Bigham et al. 2008; Dehghani et al. 2008). In this study, we also observed ectopic osteoinduction with intramuscular xenogenic DBM implantation in rats.

In this study, we focused on the levels of NO metabolites (nitrites and nitrates) during ectopic osteoinduction in rats as NO has been shown to have significant effects on osteoblasts, osteoclasts, and osteocytes. Both eNOS and iNOS have an important role in the regulation of the proliferation and function of osteoblasts (Ralston 1994; Hukkanen 1995; Riancho et al. 1995). It has also been reported that cultured osteoblasts synthesize NO in response to proinflammatory cytokines and lipopolysaccaride (Ralston 1994; Damoulis and Hauschka 1997) and that cytokine-induced NO from osteoblasts inhibits bone resorption by inducing apoptosis of osteoclast progenitor cells and suppressing the osteoclast activity (Yamamoto 1999; Mancini et al. 2000).

In terms of cell proliferation, the NOS inhibitor NG-monomethyl-L-arginine is reported to induce a dose-dependent inhibitory effect on the proliferation of

osteoblast-like cell lines MG63 and ROS 17/2.8, which indicate that NO may stimulate bone cell proliferation (Riancho et al. 1996). We propose that during the osteoinduction process local undifferentiated mesenchymal cells gradually transform into osteoblasts and resulting in a gradual increase in the levels of NO metabolites. A significant increase in NO metabolites at 6 weeks postoperatively suggested optimum activity of the osteoblasts. It is also reported that NO significantly reduced osteoblast activity at high concentration, as was evidenced by inhibition of DNA synthesis, cell proliferation, alkaline phosphatase activity, and osteocalcin production. Thus, the effect of NO on osteoblast proliferation is diverse depending on its concentration (Hukkanen 1995; Riancho et al. 1995).

Finally, according to the results of our study, ectopic osteoinduction caused increasing activity of the osteoblasts and subsequently caused increased serum levels of NO metabolites (nitrites and nitrates) due to osteoblastic activity.

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References

Bigham AS, Dehghani SN, Shafiei Z, Torabi Nezhad S (2008) Xenogenic demineralized bone matrix and fresh autogenous cortical bone effects on experimental bone healing: radiological, histopathological and biomechanical evaluation. J Orthop Traumatol 9:73–80 doi:10.1007/s10195-008-0006-6

Buttery LD, Evans TJ, Springall DR, Carpenter A, Cohen J, Polak JM (1994) Immunochemical localization of inducible nitric oxide synthase in endotoxin-treated rats. Lab Invest 71:755–764

Collin-Osdoby P, Nickols GA, Osdoby P (1995) Bone cell function, regulation, and communication: a role for nitric oxide. J Cell Biochem 57:399–408 doi:10.1002/jcb.240570305

Corbett SA, Hukkanen M, Batten J, McCarthy ID, Polak JM, Hughes SPF (1999) Nitric oxide in fracture repair: differential localization, expression and activity of nitric oxide synthesis. J Bone Joint Surg Br 81:531–537 doi:10.1302/0301-620X.81B3.8852

Damoulis PD, Hauschka PV (1997) Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. J Bone Miner Res 12:412–422 doi:10.1359/jbmr.1997.12.3.412

Dehghani SN, Bigham AS, Torabi Nezhad S, Shafiei Z (2008) Effect of bovine fetal growth plate as a new xenograft in experimental bone defect healing: radiological, histopathological and biomechanical evaluation. Cell Tissue Bank 9:91–99 doi:10.1007/s10561-008-9062-7

Diwan AD, Wang MX, Jang D, Zhu W, Murrell GA (2000) Nitric oxide modulates fracture healing. J Bone Miner Res 15:342–351 doi:10.1359/jbmr.2000.15.2.342

Farrell AJ, Blake DR, Palmer RM, Moncada S (1992) Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. Ann Rheum Dis 51:1219–1222 doi:10.1136/ard.51.11.1219

Garcia X, Stein F (2006) Nitric oxide. Semin Pediatr Infect Dis 17:55–57

Hagen JW, Semmelink JM, Klein CP, Prahl-Andersen B, Burger EH (1992) Bone induction by demineralized bone particles: long-term observations of the implant-connective tissue interface. J Biomed Mater Res 26:897–913 doi:10.1002/jbm.820260706

Hamed A, Kim P, Cho M (2006) Synthesis of nitric oxide in human osteoblasts in response to physiologic stimulation of electrotherapy. Ann Biomed Eng 34:1908–1916 doi:10.1007/s10439-006-9206-5

Hukkanen M (1995) Cytokine-stimulated expression of inducible nitric oxide synthase by mouse, rat, and human osteoblast-like cells and its functional role in osteoblast metabolic activity. Endocrinology 136:5445–5453 doi:10.1210/en.136.12.5445

Hukkanen M, Corbett SA, Batten J, Konttinen YT, Mc Carthy ID, Maclouf J, Santavirta S, Hughes SPF, Polak JM (1997) Aseptic loosening of total hip replacement: macrophage expression of inducible nitric oxide synthase and cyclo-oxygenase-2, together with peroxynitrite formation as a possible mechanism for early prosthesis failure. J Bone Joint Surg Br 179-B:467–474 doi:10.1302/0301-620X.79B3.7469

Kaur H, Halliwell B (1994) Evidence for nitric oxide-mediated oxidative damage in chronic inflammation: nitrotyrosine in serum and synovial fluid from rheumatoid patients. FEBS Lett 350:9–12 doi:10.1016/0014-5793(94)00722-5

Lincoln J, Hoyle CHV, Burnstock G (1997) Nitric oxide in health and disease. Cambridge University Press, Cambridge

Lindsay R (2004) Hormones and bone health in postmenopausal women. Endocrine 24:223 doi:10.1385/ENDO:24:3:223

Mancini L, Moradi-Bidhendi N, Becherini L, Martineti V, MacIntyre I (2000) The biphasic effects of nitric oxide in primary rat osteoblasts are cGMP dependent. Biochem Biophys Res Commun 274:477–481 doi:10.1006/bbrc.2000.3164

McClung MR (2003) Prevention and management of osteoporosis. Best Pract Res Clin Endocrinol Metab 17:53–71 doi:10.1016/S1521-690X(02)00080-5

Nathan C, Xie QW (1994) Nitric oxide synthases: roles, tolls, and controls. Cell 78:915–918 doi:10.1016/0092-8674(94)90266-6

Navarro-Gonzalvez JA, Garcia-Benayas C, Arenas J (1998) Semiautomated measurement of nitrate in biological fluids. Clin Chem 44:679–681

Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315 doi:10.1152/physrev.00029.2006

Ralston SH (1994) Human osteoblast-like cells produce nitric oxide and express inducible nitric oxide synthase. Endocrinology 135:330–336 doi:10.1210/en.135.1.330

Reddi AH, Huggins C (1972) Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc Natl Acad Sci USA 69:1601–1605 doi:10.1073/pnas.69.6.1601

Riancho JA, Salas E, Zarrabeitia MT, Olmos JM, Amado JA, Fernandez-Luna JL, Gonzalez-Macias J (1995) Expression and functional role of nitric oxide synthase in osteoblast-like cells. J Bone Miner Res 10:439–439

Riancho JA, Zarrabeitia MT, Salas E, Gonzalez-Macias J (1996) Impairment of osteoblast growth by nitric oxide synthase inhibitors: an effect independent of nitric oxide and arginine transport inhibition. Methods Find Exp Clin Pharmacol 18:663–667

Riley EH, Lane JM, Urist MR, Lyons KM, Lieberman JR (1996) Bone morphogenetic protein-2: biology and applications. Clin Orthop Relat Res 324:39–46 doi:10.1097/00003086-199603000-00006

Sakurai H, Kohsaka H, Liu M-F (1995) Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 96:2357–2363 doi:10.1172/JCI118292

Solheim E (1998) Osteoinduction by demineralised bone. Int Orthop 22:335–342 doi:10.1007/s002640050273

Stefanovic-Racic M, Meyers K, Meschter C, Coffey JW, Hoffman RA, Evans CH (1994) N-monomethyl arginine, an inhibitor of nitric oxide synthase, suppresses the development of adjuvant

arthritis in rats. Arthritis Rheum 37:1062–1069 doi:10.1002/art.1780370712

Torricelli P, Fini M, Rocca M, Giavaresi G, Giardino R (1999) Xenogenic demineralized bone matrix: osteoinduction and influence of associated skeletal defects in heterotopic bone formation in rats. Int Orthop 23:178–181 doi:10.1007/s002640050341

Ueno M, Fukuda K, Oh M, Asada S, Nishizaka F, Hara F, Tanaka S (1998) Protein kinase C modulates the synthesis of nitric oxide by osteoblasts. Calcif Tissue Int 63:22–26 doi:10.1007/s002239900484

Urist MR (1965) Bone: formation by autoinduction. Science 150:893–899 doi:10.1126/science.150.3698.893

Urist MR, Mikulski AJ, Lietze A (1979) Solubilized and insolubilized bone morphogenetic protein. Proc Natl Acad Sci USA 76:1828–1832

Urist MF, Sato K, Brownell AG (1983) Human bone morphogenetic protein. Proc Soc Exp Biol Med 173:194–199

Wimalawansa SJ, De Marco G, Gangula P, Yallampalli C (1996) Nitric oxide donor alleviates ovariectomy-induced bone loss. Bone 18:301–304 doi:10.1016/8756-3282(96)00005-1

Yamamoto S (1999) Anti-proliferative capsular-like polysaccharide antigen from Actinobacillus actinomycetemcomitans induces apoptotic cell death in mouse osteoblastic MC3T3-E1 cells. J Dent Res 78:1230–1237 doi:10.1177/00220345990780060601