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LABORATORY INVESTIGATIONS

Trigeminal Nitric Oxide Synthase Expression Correlates with New Bone Formation During Distraction Osteogenesis

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Abstract Nitric oxide synthase (NOS) has been reported to be involved with both bone healing and bone metabolism. The aim of this study was to test the null hypothesis that there is no correlation between new bone formation during mandibular distraction osteogenesis and NOS expression in the trigeminal ganglion of rats. Newly formed tissue during distraction osteogenesis and trigeminal NOS expression measured by the NADPH-diaphorase (NADPH-d) reaction were evaluated in 72 male Wistar rats by histomorphometric and histochemical methods. In animals submitted to 0.5 mm/day distraction osteogenesis, the percentage of bone tissue was higher in the basal area of the mandibles compared with the center and significantly increased through the experimental periods ($P < 0.05$). At the sixth postoperative week, the difference in bone formation between the continuous and acute distraction osteogenesis groups was the highest. Significant correlation between new bone formation by distraction osteogenesis and NADPH-d-reactive neurons was found, varying according to neuronal cell size ($r = -0.6$, $P = 0.005$, small cells strongly stained; $r = 0.5$, $P = 0.018$, large cells moderately stained). The results suggest that NOS may play a role in the bone healing process via neurogenic pathways, and the phenomenon seems to be neuronal cell morphotype-dependent. Further studies are now warranted to investigate the mechanistic link between the expression of trigeminal NOS and mandibular new bone formation by distraction osteogenesis.

Keywords Biomechanics · Bone histology and histomorphometry · Fracture repair · Nitric oxide · Orthopedics

Distraction osteogenesis is a biologic process of new bone formation between the surfaces of two bone segments, gradually separated by incremental traction [1, 2]. The distraction osteogenesis model in rat mandibles has been shown to be appropriate for investigations on new bone formation and bone healing [3–5].

Nitric oxide (NO) is considered an important signaling molecule in bone and is connected to inflammatory responses, vasoregulation, neurotransmission, neuropathic pain, sex hormones, mechanical strain, and mineralization [6–9]. It is a free-radical gas that acts as an intercellular messenger or an "atypical neurotransmitter" [10], synthesized intracellularly by NO synthase (NOS), which converts L-arginine into L-citrulline with the aid of molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor [11]. Histochemical staining for NADPH-diaphorase (NADPH-d) is a method generally accepted to detect neuronal NOS [12, 13].

Until now, the role of trigeminal NOS in bone healing has been investigated in a limited number of studies. It has been shown that bone fracture healing can be significantly improved if the surrounding tissues are supplemented with an extra dose of NO [14–16] and that organic fluid shear stress could stimulate its expression [17–19]. In an in vivo study, Davies et al. [20] observed that neuronal NOS could

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present ectopic activity at a trigeminal nerve injury site. Furthermore, possible endocrine or neurogenic pathway involvement in changes of bone mineral density and turnover has been demonstrated in neuronal NOS knockout mice [8].

The purpose of this study was to test the null hypothesis that there is no correlation between newly formed bone by distraction osteogenesis in the mandible and NOS expression in the trigeminal ganglion of rats.

Materials and Methods

In the present study, 72 male Wistar rats (300–320 g) were housed in groups of six per cage, kept in a temperature-controlled room (23–25°C), with a light/dark cycle of 12/12 hours and free access to food and water. The research protocol was reviewed and approved by the Ethics Committee for Animal Research, and all efforts were made to minimize animal suffering.

Surgical Procedures

An earlier pilot study ($n = 4$ rats) demonstrated the feasibility of a previously described distraction osteogenesis technique in the mandible of rats [3].

The animals were randomly divided into three groups of 24 subjects. After anesthesia with tribromoethanol (250 mg/kg), groups DO (distraction osteogenesis) and AD (acute distraction) had the right mandible body partially osteotomized and two titanium screws (MDT, Rio Claro, SP, Brazil) placed 2 mm from the segmented borders and linked to an orthodontic appliance (Morelli, Sorocaba, SP, Brazil) with Duralay acrylic resin (Reliance, Worth, IL). After stabilization, the osteotomy was completed, and this assembly functioned as the distractor device. Group AD had the segments separated acutely by 2.5 mm and stabilized at the end of the surgical procedure and served as a control for the distraction osteogenesis technique. In group DO, after a latency period of 7 days, the distractor was activated at a 0.5 mm/day rate for 5 days, resulting in a total lengthening of 2.5 mm (Fig. 1a). After surgery, the animals were treated with 1,200,000 U of pentabiotic (Fort Dodge, Campinas, SP, Brazil). Group NT (nontreated) had the same housing medication and feeding conditions but no surgical treatment and was the control group for NOS expression.

Eight animals per group were killed at either 2, 4, or 6 weeks from the beginning of the experiment by perfusing 100 mL 4% paraformaldehyde through their cardiovascular system, after being deeply anesthetized with urethane 37.5% (1.5 g/kg). The mandibles were dissected and reduced to smaller blocks for histologic and histomorphometric analyses. The trigeminal ganglions were removed, soaked for 2 hours in 4% paraformaldehyde in 0.1 M phosphate buffer solution, and frozen to −70°C for histochemical analysis through the NADPH-d reaction.

Histochemical Processing

Twelve sections of 40 μm from each of the bone samples of all groups were cut in a cryostat (CM1850; Leica, Heidelberg, Germany). Histologic evaluation was carried out on individual tissue sections with Nissl staining. This method, commonly used for identifying the basic neuronal structure in brain or spinal cord tissue, stains purple Nissl bodies in the cytoplasm of neurons and can be applied on formalin-fixed, paraffin-embedded tissue sections as well as frozen sections [21].

Histochemical reactions were performed according to previously reported methods [22]. NADPH-d activity was demonstrated by incubating sections in 0.1 M phosphate buffer, pH 7.4, containing 0.3% Triton X-100, 0.1 mg/mL nitroblue tetrazolium, and 1.0 mg/mL ß-NADPH at 37°C for 45 minutes in the dark. The sections were then dipped for 10 minutes in phosphate buffer, dried, rinsed in

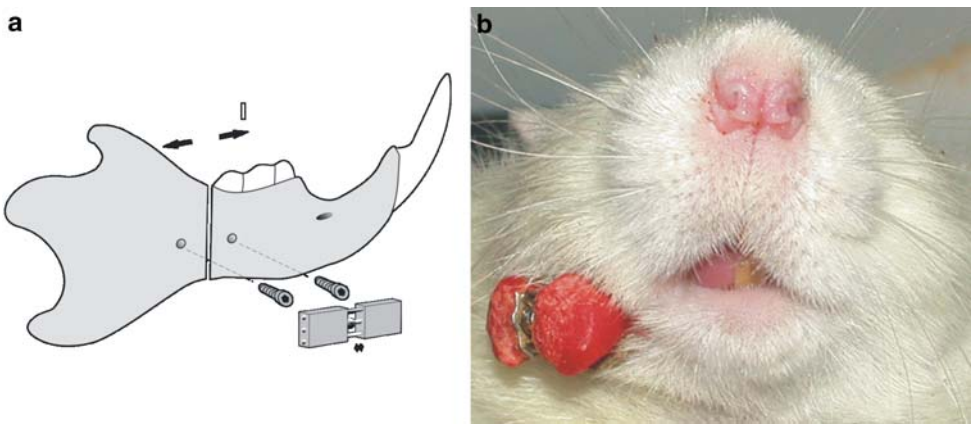

Fig. 1 (**a**) Schematic representation of the osteotomy and distractor appliance for distraction osteogenesis of the rat mandible. (**b**) Rat with distractor device showing deviation of the mandibular incisors from the median line after application of distraction osteogenesis

distilled water, dried again, and mounted for microscopic observation. NADPH-d-positive neurons could be visualized as a blue reaction product, and their location was determined using the Paxinos and Watson atlas [23].

Quantification Method

A preliminary qualitative analysis, performed by a blind examiner, identified the ganglia regions with high neuronal labeling. Positively stained neurons exhibited labeled soma, with distinct stain intensity. The number of stained neurons per ganglion, bilaterally, from an area of 100,000 μm^2, was measured using a computerized image analysis system (NIH Image System; National Institutes of Health, Bethesda, MD). Images were captured from slices using a Leica microscope, a charge-coupled device camera, and NIH Image software 9.0. They were classified and quantified according to their size (small, <20 μm; medium, 20–30 μm; large, >30 μm) and staining intensity (weakly, moderately, or strongly stained). The average number of cells stained by the NADPH-d reaction was calculated for each evaluated region and experimental period and interpreted as being representative of NOS expression.

Histologic and Histomorphometric Processing

The specimens were immersed in 4% paraformaldehyde/0.1 M phosphate-buffer solution for 24 hours, decalcified in 20% sodium citrate and 30% formic acid solutions for 15–30 days, and neutralized by a 5% sulfate sodium solution. After being embedded in paraffin, 6-μm-thick sections were cut sagittally and stained with hematoxylin and eosin and Masson's trichrome. The histologic sections of the distraction gap were observed through a grid containing 100 equidistant points, adapted to the eyepiece of a light microscope (Leica DMRB) connected to a digital camera (DP11; Olympus, Melville, NY), to measure the percentage of the new tissue components by a differential point-counting method [24]. Two thousand points in 10 rectangular fields (83,000 μm^2) lying on connective tissue and bone trabeculae were counted per animal from the central and basal region of the mandible.

Statistics

Between-group comparisons for bone formation and NADPH-d-reactive cells were performed using general linear model analysis of variance with Tukey's and Scheffe's post-hoc tests. Pearson's partial correlations between the numbers of NADPH-d-reactive neuronal cells and newly formed bone tissue, adjusted for the effect of different times of death (2, 4, and 6 weeks), were computed for each combination of neuronal cell sizes and staining intensities, which reflected the linear association between new bone formation and neuronal NOS expression. Fisher's Z statistic [25] was used to test the null hypothesis. Two-tailed $P < 0.05$ was considered statistically significant.

Results

Clinical Findings

Due to the fragility of rat mandibles, stabilization of some of the distractor devices was difficult, mainly during the first week. Five out of the 48 animals treated with mandibular distraction were replaced because the distractor device loosened (10.42%) and three because of death (6.25%). The body weight (mean ± standard deviation [SD]) of the animals submitted to surgical procedures was 313.9 ± 38.6 g at the day of surgery and 320.3 ± 63.2 g at the day of death. Clinically, a deviation of the mandibular incisors from the median line was observed in animals from groups DO and AD after complete activation of the distractor (Fig. 1b).

Histochemical, Histomorphometric, and Histologic Analyses

Although there was a higher number of stained neurons in the wounded side compared to the contralateral side of group DO, the difference was not significant ($P = 0.09$). Examples of NADPH-d-reactive neuron morphotypes are illustrated in Figures 2 and 3.

In the trigeminal ganglia of group DO, the number of small cells strongly stained by the NADPH-d reaction

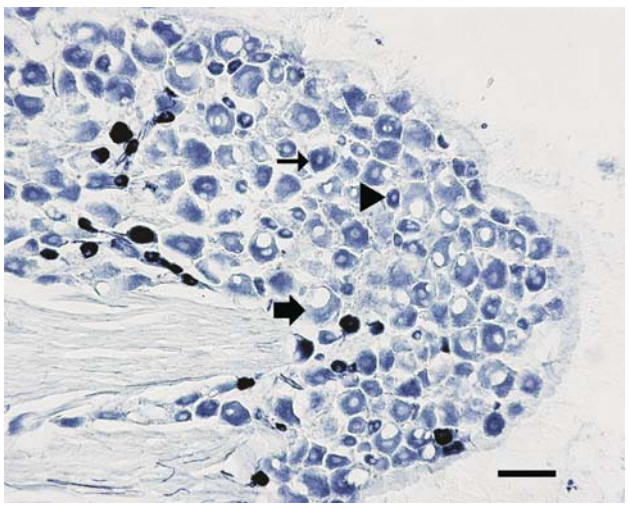

Fig. 2 Small (*arrowhead*), medium (*thin arrow*), and large (*thick arrow*) NADPH-d-reactive trigeminal neuronal cells from group DO after 4 weeks of consolidation. Scale bar = 50 μm

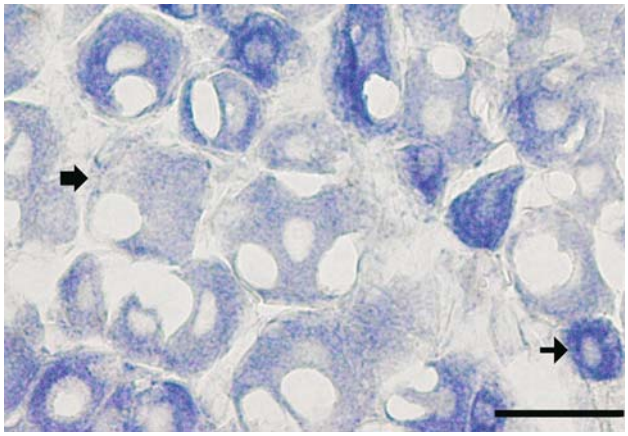

Fig. 3 Small strongly stained (*thin arrow*) and large weakly stained (*thick arrow*) trigeminal neuronal cells from group DO after 2 weeks of consolidation. Scale bar = 50 μm

(11.23 ± 5.5, mean ± SD) was significantly higher in comparison with group NT (8.25 ± 3.29, $P < 0.05$; Table 1). There were no significant differences between the numbers of median and large cells strongly stained from groups DO and NT. Median strongly stained cell counts were 8.44 ± 4.39 in group DO and 7.12 ± 4.26 in group NT. Large strongly stained cell counts were 5.02 ± 1.93 and 5.23 ± 1.54 for groups DO and NT, respectively.

In the DO group, a higher percentage of new bone tissue was found in the basal region compared to the center of the mandible and the basal bone counts significantly increased between the 2-week periods ($P < 0.05$, Table 2). At the sixth postoperative week, the difference in bone formation between groups DO and AD was the highest, although statistically not significant ($P = 0.08$ for treatment factor).

More organized and mineralized new bone tissue was observed in the distraction osteogenesis group after the fourth and sixth postoperative weeks, when almost complete closure of the distraction gap was seen in approximately 90% of histologic slices (Figs. 4, 5a). Fibrous connective tissue mixed with small and immature trabeculae surrounded by osteoblasts growing toward the center of the lesion was also present (Figs. 5a, 6). The areas of fibrous tissue were apparently larger in animals treated with acute distraction and frequently extended to the bone cortex (Fig. 5b).

Correlation between Bone Formation and NADPH-d-Reactive Neurons

Significant correlation between new bone formation by distraction osteogenesis and NADPH-d-reactive neurons was found, which varied according to neuronal cell size and staining intensity (Fig. 7). New bone formation varied inversely to the numbers of small cells moderately stained ($r = -0.44$, $P = 0.03$) and of small cells strongly stained ($r = -0.56$, $P = 0.005$). In contrast, there was a positive correlation between new bone formation and medium cells weakly stained ($r = 0.48$, $P = 0.02$) and large cells moderately stained ($r = 0.48$, $P = 0.02$).

Discussion

NO is an important regulator of bone metabolism produced through activation of the NOS enzyme. The null hypothesis that there is no correlation between new bone formation by mandibular distraction osteogenesis and NOS expression in

Table 1 Means (SD) number of small, medium and large trigeminal neuronal cells per 100,000 μm^2 according with their staining intensity by the NADPH-diaphorase reaction from groups 1 and 3 ($n = 24$)

Cell size	Group DO			Group NT (control)		
	Weakly stained	Moderately stained	Strongly stained	Weakly stained	Moderately stained	Strongly stained
Small						
2 weeks	12.54 (2.77)	11.64 (5.16)	10.10 (2.60)*	10.01 (3.02)	11.69 (3.51)	9.68 (3.08)*
4 weeks	8.73 (4.03)	10.16 (3.89)	12.33 (8.82)*	9.33 (2.69)	8.64 (3.09)	6.39 (2.32)*
6 weeks	7.19 (1.96)	10.56 (3.72)	11.24 (3.45)*	9.01 (3.01)	8.49 (2.73)	8.68 (3.76)*
Medium						
2 weeks	17.23 (5.89)	14.89 (3.16)	5.60 (1.26)	15.16 (3.21)	16.47 (5.10)	8.02 (3.15)
4 weeks	16.28 (5.94)	15.08 (4.79)	9.06 (5.05)	16.30 (3.61)	16.80 (4.74)	6.30 (3.47)
6 weeks	13.17 (4.54)	15.44 (4.66)	10.66 (4.58)	13.27 (4.34)	14.77 (3.08)	7.04 (5.99)
Large						
2 weeks	5.99 (1.52)	3.41 (0.80)	0.89 (0.33)	4.79 (1.25)	2.75 (1.56)	0.67 (0.43)
4 weeks	5.11 (2.42)	3.73 (1.22)	0.75 (0.69)	5.63 (1.93)	4.11 (1.15)	1.12 (0.69)
6 weeks	3.96 (1.30)	4.36 (1.03)	1.67 (0.93)	5.28 (1.46)	3.92 (1.06)	1.14 (0.61)

Overall mean numbers of small strongly stained cells differed between groups 1 and 3 (*$P < 0.05$)

Table 2 Mean (SD) percentage of bone tissue counts from the basal and central area of the distraction osteogenesis site in groups 1 and 2 ($n = 24$)

Subgroups	Group DO		Group AD (control)	
	Center[a]	Base[a]	Center	Base
2 weeks	27.22 (12.97)	54.54* (16.51)	32.03 (17.19)	48.02 (23.07)
4 weeks	37.02 (16.27)	69.97* (19.35)	47.72 (17.60)	59.78 (23.04)
6 weeks	41.25 (26.16)	80.37* (13.97)	42.16 (19.84)	66.03 (26.09)

[a], * $P < 0.05$ (Tukey test)

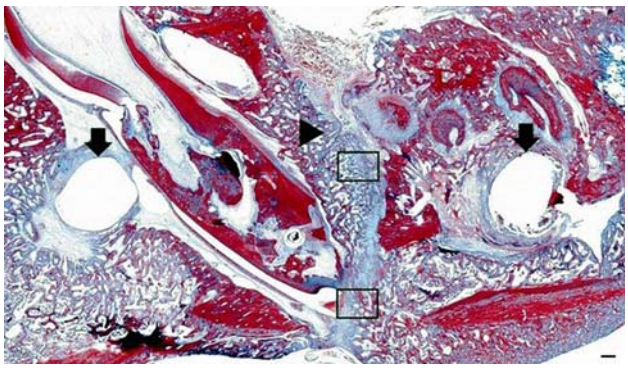

Fig. 4 Sagittal Masson's trichrome-stained section from the distraction osteogenesis group, 4 weeks after 2.5 mm activation and stabilization of the distractor device: *arrowhead*, new formed tissue; *downward arrow*, position of screw; *rectangles*, the basal and central regions evaluated. Scale bar = 500 μm

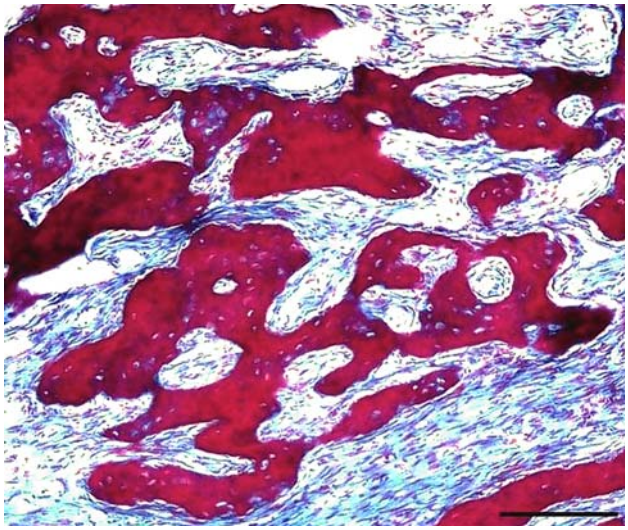

Fig. 6 Masson's trichrome–stained section from group DO. Central area of the mandible, showing connective tissue and newly formed bone surrounded by osteoblasts after 4 weeks of stabilization. Scale bar = 100 μm

the trigeminal ganglion was tested using an experimental model for distraction osteogenesis in rats. We found evidence of correlation between the content of NADPH-d/NOS present in the trigeminal ganglion neurons and bone healing in rat mandibles. Furthermore, this correlation was cell morphotype-dependent and more pronounced among the smaller neuronal cells. Therefore, the null hypothesis was rejected.

It is known that NO released from bone cells is associated with cellular mechanotransduction, exerting a strong inhibitory effect on osteoclast activity, whereas suppression of NOS can impair wound healing, angiogenesis, bone mineralization, and turnover [8, 26]. In the present study, the variation of NOS expression was investigated in trigeminal cells during the bone healing process and found to be significantly increased in the "small" cell morphotype. Although this is in contrast with a previous report of reduced NOS expression after inferior alveolar nerve injury in ferrets [20], other studies have found an increased concentration of NOS in the trigeminal and dorsal root ganglia following submandibular branch transaction and sciatic nerve injury, respectively [27–30]. The correlation is also

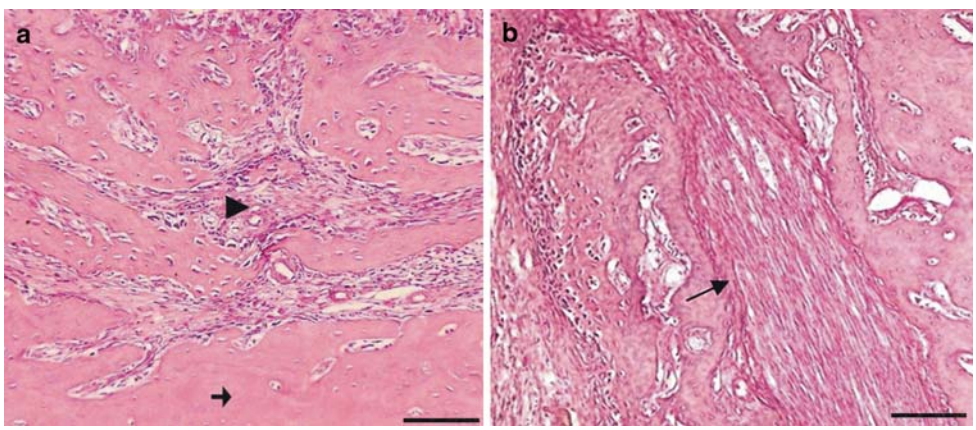

Fig. 5 Hematoxylin and eosin–stained sections of the healing site after 6 weeks of stabilization. (**a**) Basal (*arrow*) and central (*arrowhead*) areas of mandible from group DO. (**b**) Extensive area of fibrous connective tissue (*arrow*) from group AD. Scale bar = 100 μm

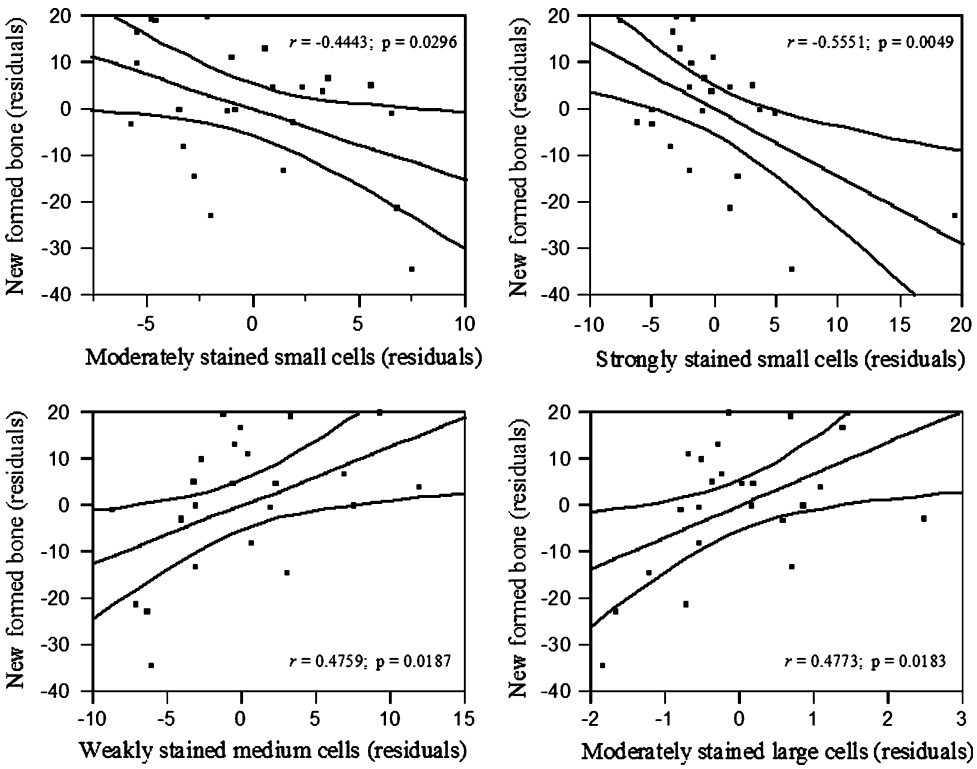

Fig. 7 Correlations (r) between mean percentages (residuals) of new formed bone tissue by distraction osteogenesis and mean numbers (residuals) of NADPH-d-reactive neuronal cells in the trigeminal ganglion from group DO ($n = 24$)

in accordance with the increased bone mineral density and decreased bone turnover in neuronal NOS knockout mice recently described and considered attributable to a systemic endocrine or neurogenic pathway [8]. The less intense but persistent effect in the contralateral trigeminal ganglion suggested the presence of signaling mechanisms linking the two sides of the body [27, 31, 32].

Surgical procedures and bone lengthening might have generated pain to which the animals responded with movement restriction favoring stability of the bone segments and tissue differentiation toward healing. It is likely that these sensory and efferent responses also influenced NOS expression in the trigeminal nerve as NO has been implicated in pain mediation and control of jaw reflexes and movements [33–35], other than with a local response from bone cells. Another possible participation of neuronal NOS (nNOS) on the healing process is via axonal transportation. Accumulation of nNOS extending 1 mm at an inferior alveolar nerve injury site has previously been detected, indicating a possible translocation of the neuronal enzyme from the cell body to the site of injury [20, 36, 37].

Since the number of small cells expressing NOS correlated inversely with bone formation while the correlation between the number of medium and large cells was direct, we speculate that distinct cell morphotypes may influence the healing process by different mechanisms. The correlation of about 0.5 suggests that NOS may participate in the bone healing process along with other factors. However, it was not possible to estimate how much the ganglion trigeminal NOS either directly or indirectly accounted for new bone formation. The strictly correlative nature of these data did not allow testing for a mechanistic link between NOS expression and bone formation.

The rat model used for distraction osteogenesis proved to be viable and simple, although some of the devices were lost during the experimental period, mainly due to loosening of the titanium screws. Although the differences between groups DO and AD did not reach statistical significance ($P = 0.08$), the distraction osteogenesis technique showed a tendency to produce higher percentages of new bone tissue. We accredited this nonsignificant difference to the 2.5 mm gap created after complete activation of the distractor device, which was possibly too small. A later study has shown that full-thickness acute defects of up to 5 mm in Wistar rat mandibles can potentially self-repair [38]. Hence, bony formations in addition to fibrous tissue and cartilage seen in the distraction gap of some animals treated with acute distraction may have occurred due to the proximity of the mandibular segments. On the other hand, lack of complete stability of the distractor device observed in some animals may have increased tissue response variability.

In conclusion, the distraction osteogenesis model in rat mandibles demonstrated feasibility as a method to study new bone formation. NOS expression by neuronal trigeminal cells measured through the NADPH-d reaction correlates with new bone formation by distraction osteogenesis in rat mandibles, and this phenomenon seems to be

cell morphotype-dependent. Further studies are now warranted to investigate the mechanistic link between the expression of trigeminal NOS and mandibular new bone formation by distraction osteogenesis.

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