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ORIGINAL ARTICLE

Dominique Seux · Annick Roméas · Bruno Antoine · Henry Magloire · Françoise Bleicher

In vitro study of a neodynium:yttrium aluminum perovskite laser on human nonexposed pulp after cavity preparation

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Abstract *Purpose:* The aim of this study was to investigate dental pulp reactions after a neodynium:yttrium aluminum perovskite laser pulse on the dentinal floor of occlusal cavities in an in vitro model. *Methods:* A Lokki dt laser was used at 30 Hz, 5 W, and 160 mJ for 0.5 s. The pulp reactions were analyzed in a previously described human tooth slice cultured model. The following markers were identified by immunohistochemistry: collagens I, III, and IV and HLA-DR-positive cells. *Results:* After 4 days of culture, under laser pulse, a concentration of type III collagen beneath the odontoblast layer, a higher level of vessels and an accumulation of HLA-DR-positive cells were routinely observed subjacent to the cavity. *Conclusion:* This laser treatment leads to the first step of rapid pulp repair under culture conditions.

Keywords Cavity preparation · Neodynium:yttrium aluminum perovskite laser · Pulp repair · Immune response · Immunohistochemistry

Introduction

During carious processes or cavity preparation, the dentine/pulp complex attempts to protect itself by defense reactions including (1) the involvement of pulpal dendritic cells (the most critical antigen-presenting cells) implicated in the primary immune responses [10], and (2) the production of tertiary dentine (reactionary or reparative dentine) [18]. If left untreated, pulp inflammation will ensue: pulpitis develops through the tissue [7], whereas it is localized and reversible at the beginning of the process [3]. Until now, the treatment was restricted to classic endodontic therapy [4].

D. Seux · A. Roméas · B. Antoine · H. Magloire (✉) · F. Bleicher
Laboratoire du Developpement des Tissus Dentaires,
Faculté d'Odontologie,
rue G. Paradin, 69372 Lyon cedex 08, France
e-mail: magloire@laennec.univ-lyon1.fr
Tel.: +33-4-78778685
Fax: +33-4-78778757

In considering vital pulp therapy, a distinction must be made between pulpal nonexposed and exposure situations. The treatment of vital exposed pulp by direct pulp capping with calcium hydroxide has a long history and has been well documented [18]. Generally, it induces dentinal bridge formation to promote successful healing. In case of vital closed pulp, new treatment strategies have been introduced in addition to traditional indirect pulp capping materials. It was recently shown that dentine matrix components can stimulate reactionary dentinogenesis in nonexposed cavity preparation by transdentinal stimulation [20, 26]. However, this process is not yet used at the human level.

Another method to maintain dental pulp vitality is the use of laser radiation on the floor of the cavities after caries removal. Until now, lasers have been mainly used for carious tissue removal and cavities preparation. Carbon dioxide and neodynium:yttrium aluminum garnet (Nd:YAG) lasers were the first ones used in dentistry. However, one of the major problems (or disadvantages) was a markedly elevated intrapulpal temperature [27]. In contrast, erbium:yttrium aluminum garnet (Er:YAG) laser produced minimal damage to pulp tissues and stimulated pulp repair processes [23, 24]. Similarly, the neodynium:yttrium aluminum perovskite (Nd:YAP) fiber laser type has been successfully used in endodontic treatment, providing an antibacterial effect by vaporization of residual organic tissue in the root canal [2, 16]. Thus, it was tempting to use this kind of laser beam through the residual layer of dentine to remove the localized injured pulp zone and stimulate pulp tissue repair without significant dentinal or pulpal damage.

The purpose of this study was to investigate in vitro the pulpal reactions following Nd:YAP laser irradiation on dentine cavity preparation. We used the thick-sliced human teeth cultivated in vitro model previously described [14].

Materials and methods

Preparation

Thirty-six fresh, noncarious, human third molar teeth were extracted from patients 18–40 years old for orthodontic reasons with the informed consent of the patients in accordance with French legal requirements. Immediately after extraction, they were put in the culture medium before being used. Radiography was done to visualize the extent of the pulp tissue. An occlusal cavity was prepared on each tooth with a diamond bur (1.6 mm diameter) under water-spray cooling. The size of these cavities was standardized, so that they did not extend over more than one third of the dentine thickness.

Laser procedure

The pulse Nd:YAP laser apparatus (1.34-μm wavelength) used in this study was a Lokki dt (Lokki, Vienne, France) [12].The pulse duration was 150 μs. The laser could provide repetition rates of 0.5 s to 2 s, an energy of 100 mJ to 400 mJ per pulse, and frequencies of 5 Hz, 10 Hz, or 30 Hz. Thus, several operating modes were available. The apparatus has an optic fiber with two possible external diameters (240 μm and 385 μm). The former was chosen for the experiments below.

Light microscopy

This preliminary study was performed to determine the specific parameters for laser use as a determinant of microscopic dental pulp modifications. Twelve freshly extracted noncarious human third molars were selected. Immediately after cavity preparation, they were divided into three experimental groups. The dentine floor was irradiated using three operating modes, respectively, with different frequency/power/energy based on previous pilot data given by the manufacturers: group 1 (D+) 10 Hz at 400 mJ and 4 W, group 2 (G+) 30 Hz at 330 mJ and 10 W, and group 3 (G–) 30 Hz at 160 mJ and 5 W. Each mode was performed on the floor of each cavity for 0.5 s.

Controls were performed on two untreated teeth (without cavity and laser irradiation) and on two teeth with cavities. Morphological analysis was routinely performed on demineralized specimens previously fixed in Bouin's fluid and embedded in paraffin. The slices were then stained with Masson trichrome.

The in vitro model

Twenty-two freshly extracted noncarious human third molars were selected for this study. Thick slices, with cavity only and laser pulse (G–) in the depth of the cavity, were performed as previously described [14]. Briefly, three or four thick slices (750 μm) per tooth were carefully obtained with a sawing machine (Isomet Buehler, Evanston, Ill., USA) under culture medium flow. One slice of each tooth was fixed immediately in 4% paraformaldehyde in phosphate buffer as control. The other slices were placed in 12-well Falcon culture plates (Becton Dickinson, Oxford, UK) and covered with 1 ml of Eagle's basal medium (Gibco, Grand Island, N.Y., USA) containing 50 mg/ml of ascorbic acid, 10% fetal calf serum (Roche, Mannheim, Germany), and antibiotics. The slices were cultured for up to 4 days.

Immunohistochemistry

Cultures of undemineralized tooth slices were prepared according to the protocol previously described [13]. The slices were quickly rinsed in phosphate-buffered saline (PBS), then fixed in 4% paraformaldehyde/PBS solution for 1 h at 4°C. The slices were washed in PBS and immersed in 7% saccharose/PBS, then in 15% saccharose/8% glycerol/PBS. The hard tissue was gently removed; then the pulpal tissue was embedded in Tissue Tek compound (EMS, Washington, Penna., USA) and immersed in liquid nitrogen-cooled isopentane. Cryostat serial sections (10 μm) were collected on 3-aminopropyltriethoxysilane-coated slides and air-dried. Immunofluorescence labeling (for collagens I, III, and IV) and immunoperoxidase ABC Vectastain (for HLA-DR) were routinely used. For fluorescence, cryostat sections (10 μm) were rinsed in PBS, incubated in 0.2% bovine serum albumin and normal goat serum (dilution 1/50) PBS, then with specific polyclonal antibodies (anticollagen I) (Novotec, Lyon, France, ref. 20–111, dilution 1/40) or monoclonal antibodies (anticollagen III, Novotec, ref. 8D5, 1/20 and anticollagen IV, Novotec, ref. 24D1, 1/80). For immunoperoxidase, after rehydration in PBS, the sections were treated with 0.3% H_2O_2-methanol to inhibit endogenous peroxidases, rinsed in PBS buffer prior to application of primary antibodies (anti-HLA-DR clone LN3 B, 1/50) (Zymed, San Francisco, Calif., USA), and then treated with mouse Vectastain Elite ABC Kits using the diaminobenzidine peroxidase substrate kit according to the manufacturer's protocol (Vector, Burlingame, Calif., USA).

Results

Preliminary histologic results

In control teeth without cavity (C–) and without laser pulse (L–), the odontoblast layers and dental pulp tissue appeared to be normal (Fig. 1a). After cavity preparation (C+) without laser pulse (L–), a slight disorganization of the odontoblast layers was observed (Fig. 1b). After one laser pulse (G–) in the cavity, most of the odontoblasts adhered to the dentine wall (Fig. 1c), but some cells appeared to be disturbed subjacent to the cavity (injured zone). After cavity preparation and one G+ pulse, separation of the odontoblast layer from the predentine was observed beneath the cavity (Fig. 1d). After cavity preparation and a D+ pulse, extensive disorganization of the odontoblast layer with microabscess was detected, affecting the complete zone (Fig. 1e). Taken together, these data prompted us to use the G– beam only during the in vitro experimentation.

Immunodetection of collagens I, III, and IV

At T0, no differences in terms of staining intensity or distribution patterns of type I collagen were found with or without laser pulses. Immunolocalization was observed in the odontoblast layer, predentine, dentine, and pulp tissue (Fig. 2a, b). After 4 days of culture, a strong immunoreactivity of type I collagen was seen in the odontoblast layer beneath the cavity in both groups (Fig. 2c, d). At T0, the type III collagen staining was localized under the odontoblast layer in the pulp core. No reactivity was detected in the dentine. These observations were found with and without laser pulse (Fig. 2e, f). After 4 days of culture, no modification could be seen without laser beam (Fig. 2g). Under laser, beneath the injured zone, type III collagen was concentrated under the odontoblast layer (Fig. 2h). At T0, immunolocalization of type IV collagen was specifically observed around vessels with and with-

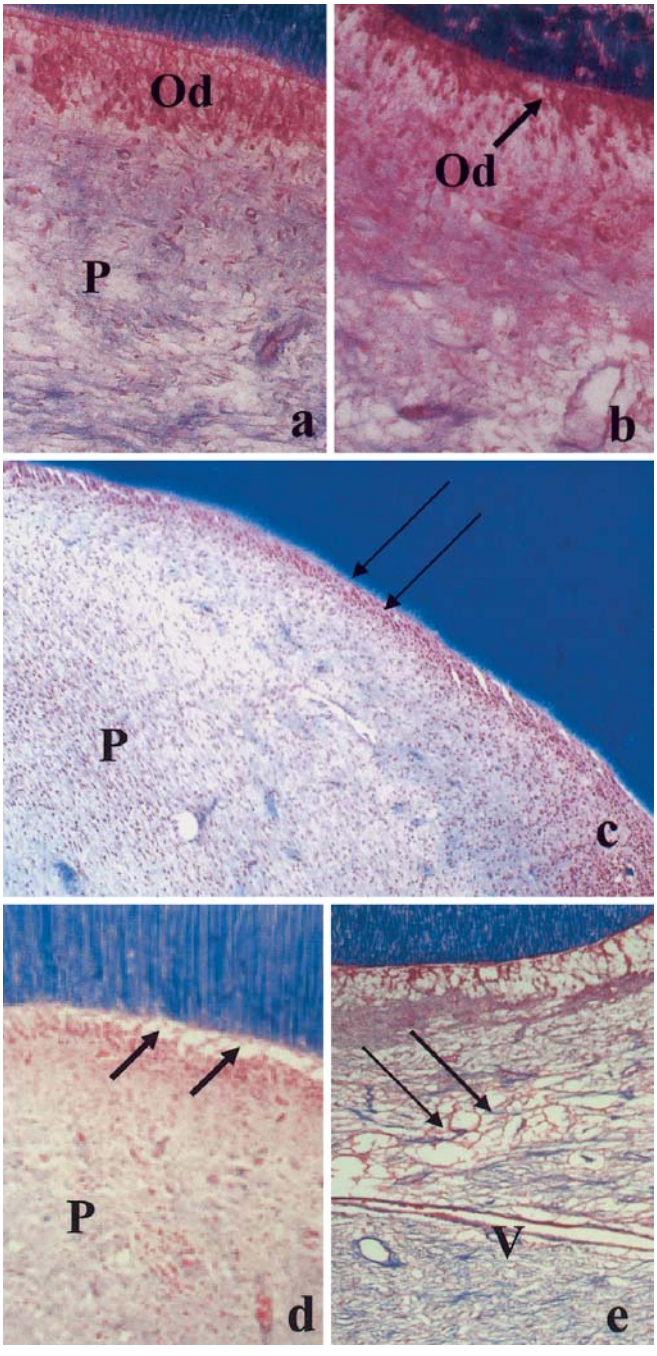

Fig. 1 Morphological study on demineralized teeth of untreated and treated teeth just after extraction (T0). Masson trichrome staining. *Od* odontoblast, *P* pulp, *V* vessel, *arrows* microabscess. **a** Control tooth without cavity (C–) and without laser pulse (L–) (×150). **b** Control tooth with cavity (C+) and without laser pulse (L–) (×150). **c** C+ and first operating mode (G–) (×80). **d** C+ and second operating mode (G+) (×150). **e** C+ and third operating mode (D+) (×140)

out laser pulse (Fig. 2i, j). After 4 days, with laser pulse on the floor of the cavity, a higher density of vessels was observed, including pulp horns (Fig. 2k, l). In negative control, no specific labeling was observed (data not shown).

Immunodetection of dendritic cells

At T0, the HLA-DR-positive dendritic cells were detected in the whole pulp core and in the subodontoblastic layer with or without laser pulse (Fig. 3a, b). After 4 days of culture, no changes could be observed without laser beam (Fig. 3c). In contrast, under laser pulse, a linear accumulation of HLA-DR-positive dendritic cells was noted in the subjacent tissue close to the cavity (Fig. 3d), around the vessels (Fig. 3e), and along the nerve fibers (Fig. 3f). No specific labeling was observed on negative control (Fig. 3g).

Discussion

The present study shows that a Lokki pulse Nd:YAP laser apparatus focused on the dentinal floor after cavity preparation can be successfully used without damage to the pulp tissue on extracted teeth. In addition, no significant pulp reactions could be observed between the laser-free and -treated groups at a frequency of 30 Hz and energy of 160 mJ during 0.5 s. Under these specific conditions and following cavity preparation and laser pulse cultured for 4 days, the sliced teeth showed the first steps of pulp repair: type III collagen expression, neovascularization, and dendritic cell concentration in the affected zone.

It is well known that cavity preparation procedures have a significant influence on healing processes [17, 19]. In our in vitro study, the preparation of occlusal cavities with a high-speed handpiece and procedures preventive of histological change (e.g., adequate cooling of the high-speed bur) induced mild and localized pulp modifications as described previously in vivo [11, 22]. Under these conditions, this well controlled and reproducible procedure was successfully used in our experiments.

Previous reports indicate that cavity preparation with Er:YAG laser irradiation induced minimal, reversible pulp modifications similar to those from a high-speed handpiece [21]. A Nd:YAP laser raised temperatures significantly more than Er:YAG or handpiece use during ablation of dentine [1]. Thus, before conducting the in vitro study on the pulpal reactions to the Lokki laser, a preliminary study was made to determine the specific parameters for using this new Nd:YAP laser after cavity preparation with a high-speed handpiece. We observed no significant difference in pulp reaction between the laser-free and -treated groups with our laser generating an energy of 160 mJ and with a pulse repetition rate of 30 Hz. The response of the nonexposed dental pulp to the laser beam was mild and localized. Thus, the parameters corresponding to this group (C+, G–) seemed to be adequate for studying human nonexposed pulp reactions after cavity preparation. These results were very similar to the pulp reactions obtained with an Er:YAP laser or high-speed handpiece [8, 23].

Thick-sliced human teeth cultivated up to 4 days in vitro can simulate the onset of reparative dentine forma-

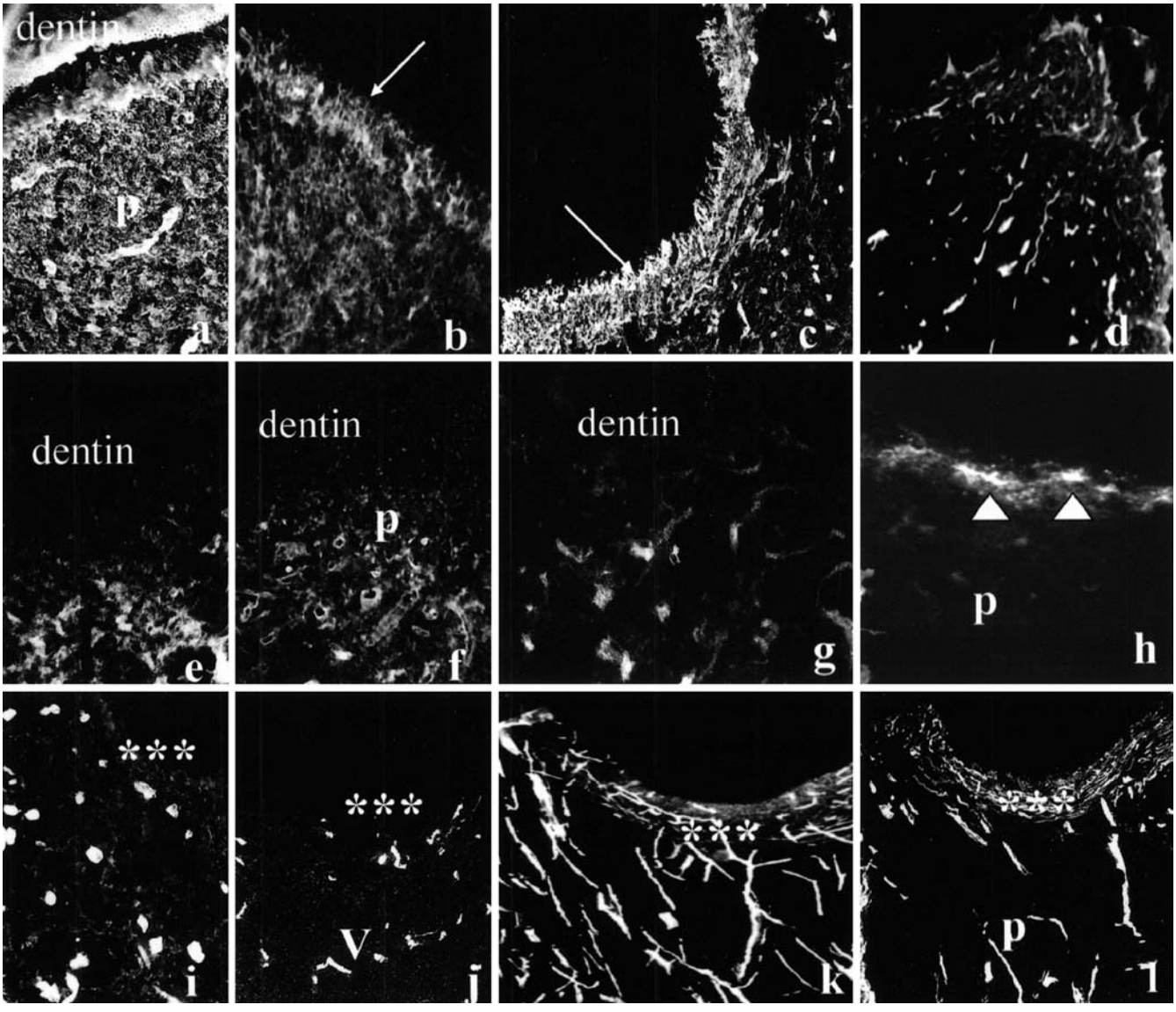

Fig. 2 Immunohistochemical localization of types I, III, and IV collagens in the pulp tissue beneath the dentine cavity (C+), without (L–) or with laser pulse (G–), immediately after cavity preparation (T0) or cultured for 4 days (J4). *V* vessel, *p* pulp. **a** C+, L–, T0 (×130). **b** C+, G–, T0. *Arrow* type I collagen (×100). **c** C+, L–, J4. *Arrow* type I collagen (×50). **d** C+, G–, J4 (×40). Type III collagen (*triangles*). **e** C+, L–, T0 (×130). **f** C+, G–, T0 (×130). **g** C+, L–, J4 (×130). **h** C+, G–, J4 (×130), type IV collagen (***). **i** C+, L–, T0 (×130). **j** C+, G–, T0 (×409). **k** C+, L–, J4 (×40). **l** C+, G–, J4 (×40)

tion under operative procedures [6, 13, 14, 15]. Interestingly, under laser irradiation after 4 days in culture only, significant events characterizing a rapid pulp repair process were observed compared to laser-free teeth: a marked increase in metabolic activity for type III collagen (concentrated under the odontoblast layer) and sprouting of blood vessels (a higher concentration of type IV collagen) were particularly identified. In vitro, this laser treatment leads to the first step of a faster pulp repair process than after high-speed drill alone, similar to cavity preparation using an Er:YAG laser [23, 24]. In addition, the application of a YAP laser beam generates a more marked accumulation of HLA-DR-positive dendritic cells at the pulp/dentine border beneath the injured zone than with laser-free cavity preparations.

It is well known that these immunocompetent, HLA-DR-positive dendritic cells, essential for the initiation of immune responses, play a central role in the process of pulpal healing [6, 7, 9, 28]. Therefore, it seems likely that a specific stimulating process could occur, suggesting a new positive role of laser application for pulp repair. Very recently, it has been demonstrated in the same culture model [6] that transforming growth factor beta 1 (TGF-β1), involved in the induction of cicatricial dentine production [25] and the immune response [5], was capable of inducing the accumulation of dendritic cells in odontoblast and subodontoblastic layers. In this con-

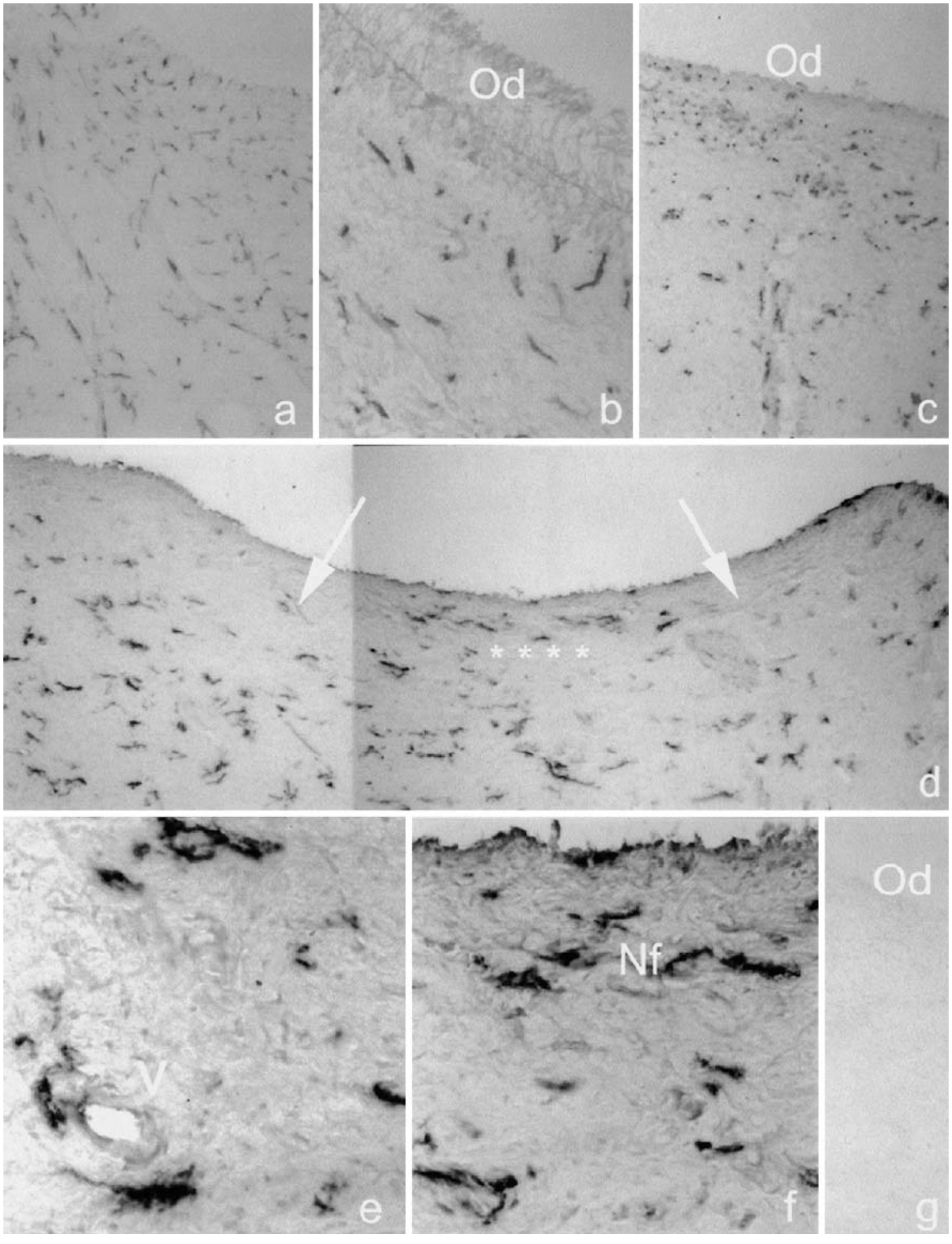

Fig. 3 Immunoperoxidase staining of HLA-DR-positive dendritic cells in the pulp tissue at T0 or cultured for 4 days (J4). *Od* odontoblast, *V* vessel, *Nf* nerve fiber. **a** C+, L–, T0 (×75). **b** C+, G–, T0 (×230). **c** C+, L–, J4 (×75). **d** C+, G–, J4. Dendritic cell density was increased in odontoblast and subodontoblastic layers beneath the dentine cavity (****) but not in the pulp horns (*arrows*) (×75). Note HLA-DR-positive dendritic cells around vessels (**e**) and along nerve fibers (**f**) (×300). **g** Negative control (×230)

text, it is tempting to imagine that TGF-β1 or other active molecules could be released from laser-treated dentine tissue to diffuse to pulp tissue and trigger the cascade of events giving rise to rapid pulp repair.

Under routine Nd:YAP laser irradiation, the thermal effect was previously demonstrated to be determinant [1] with 240 mJ and a pulse repetition rate of 10 Hz. Under the minimal conditions used in this experiment (160 mJ,

one pulse), it seems likely that the thermal damage to adjacent tissue was strongly reduced, thus allowing a clinical application to stimulate pulp healing. Therefore, in case of localized and reversible pulpitis [3], removal of the injured zone and laser stimulation of pulp tissue repair through the dentine layer could successfully be used in vital therapy.

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