Immediate Placement of Implants into Periodontally Infected Sites in Dogs. Part 2: A Fluorescence Microscopy Study

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Purpose: *Polychromatic sequence labeling of bone was used to study the effect of periodontal infection on the immediate placement of Frialit-2 implants.* ***Materials and Methods:*** *In the surgical first phase, periodontitis was induced with ligatures involving the mandibular premolars of 5 mongrel dogs, and the contralateral teeth were used as controls (received only prophylaxis). After 3 months, the second phase was initiated and 40 implants were placed in the alveoli of both experimental and control teeth. During the healing period, fluorescent bone markers were injected to study bone formation around the implants. The dyes were injected in the following sequence: oxytetracycline hydrochloride at 3 days after implant placement, calcein green 4 weeks after implant placement, oxytetracycline 8 weeks after implant placement, and alizarin red S 3 days before sacrifice. Following a healing period of 12 weeks, the animals were euthanized and the hemimandibles were removed, dissected, fixed, and prepared for histomorphometic analysis of the percentage of each bone marker present.* ***Results:*** *Fluorescence microscopy showed a similar sequence of bone remodeling (Mann-Whitney test) for both groups: experimental group, 9% bone formation at 3 days, 29% at 4 weeks, 21.6% at 8 weeks, and 52% at 12 weeks; control group, 14% at 3 days, 35.2% at 4 weeks, 32.3% at 8 weeks, and 45.8% at 12 weeks.* ***Discussion:*** *Remodeling in both groups had similar characteristics in the degree of bone formation.* ***Conclusions:*** *It was concluded that periodontal disease does not affect bone remodeling around immediate implants. Although the healing in periodontally infected sites was slower initially, it reached the levels of the non-diseased sites after 12 weeks.* INT J ORAL MAXILLOFAC IMPLANTS 2003; 18:812–819

Key words: *dental implants, endosseous dental implantation, fluorescence microscopy, periodontitis*

Fluorescence is defined as the property possessed by certain substances of converting short wavelengths of light into radiation of longer visible wavelengths.[1] Primary fluorescence (autofluorescence) is the inherent capacity of substances to fluoresce when exposed to an exciting ultraviolet light source. Secondary fluorescence is the fluorescence induced in substances by the application of fluorescent compounds or dyes (fluorochromes). Several bone-labeling fluorochromes are used in bone studies, namely hematoporphyrine, 2,4-bis [N,N'-di(carbomethyl) aminomethyl] fluorescein[2,3] (DCAF), calcein blue,[4] xylenol orange,[5] calcein green,[6] synthetic alizarin red complexone,[7,8] and tetracycline.[9–11] These markers provide important information relative to the physiologic history, bone formation rate, thickness and volume of bone formed per unit of time, and percentage of new bone for diagnostic or research purposes when applied in vivo.

Remodeling of compact bone adjacent to implants results in the formation of secondary osteons, which are concentric lamellar structures with a peripheral scalloped margin. When bone remodels adjacent to an implant, this cementing

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substance may provide mechanical adhesion of bone at the implant surface.[12] Interface development has been described by Roberts[12] as occurring in the following sequence:

1. *Woven callus.* Following implantation of a biocompatible device into cortical bone, a bridging callus forms at the periosteal and endosteal surfaces. Under optimal conditions (minimal trauma and vascular compromise), the callus originates within a few millimeters of the margin of the implantation site.
2. *Lamellar compaction.* The lattice structure of the callus provides space for lamellar bone. The lattice is filled with well-organized lamellae and the resulting composite bone formed is relatively strong.
3. *Interface remodeling.* In this phase, remodeling is achieved in a nonvital interface by cutting/filling cones emanating from the endosteal surface. The mechanism is similar to typical cortical remodeling except that the cutting/filling cones are oriented perpendicular to the normal pathway (long axis of the bone). The fluorochrome markers in the present study were evaluated at up to 12 weeks of this stage.
4. *Maturation.* The compact lamellar bone matures through a series of modeling and remodeling processes, until the callus is completely resorbed (modeling).

It has been suggested that immediate implant placement is contraindicated in the presence of periapical and periodontal lesions.[13–17] However, in histomorphometric evaluations of immediate implantation in dogs with induced periapical lesions[18] and with periodontally infected sites,[19] investigators reported that osseointegration occurred in both experimental and control sites. According to these studies, immediate implant placement in the presence of periapical endodontic lesions or periodontally infected sites is not contraindicated if appropriate therapy is carried out and if adequate care is taken both preoperatively and postoperatively.

In this study, the chronology of bone remodeling adjacent to the bone-implant interface was evaluated by fluorescence microscopy to confirm these results, using 3 different bone markers. The role of periodontal infection at the time of immediate implantation and during all phases of healing, and the issue of whether there was a difference between nonthreaded and threaded areas of the implant, were evaluated. The behavior of the mineralized tissue during healing and the usefulness of fluorescence microscopy for this analysis were also studied.

MATERIALS AND METHODS

Five young adult male mongrel dogs, weighing approximately 10 kg, were used in this study. The animals had intact maxillae and atraumatic occlusion. They had no oral viral or fungal lesions, and they were in good general health with no systemic involvement. The procedures were in accordance with guidelines approved by the Council of the American Psychological Society (1980) for animal experiments.

The dogs were not fed the night before the surgical procedure. They were sedated and then anesthetized with 1 mL/kg Thiopental (Cristália Laboratory, Itapira, SP, Brazil), 20 mL diluted in 50 mL saline administered intravenously.

Surgery was carried out by quadrants in each animal as described by Novaes and coworkers.[19] In the mandibular first, second, third, and fourth premolar sites on the control side, the teeth received prophylaxis only; the contralateral side served as the experimental group, where periodontitis was induced according to the technique of Schliephake and Kracht.[20] In summary, a nonresorbable silk suture was placed into a 1-mm-deep infrabony pocket that was created around each premolar after dissection of the marginal periodontium. The suture was left in place for 3 months on the experimental side. After confirmation of periodontal infection by the presence of soft tissue and radiographic images of bone loss and furcation involvement, all experimental and control side premolars were extracted without damaging the bony walls.

After confirmation of periodontal disease at the experimental sites, the animals were anesthetized in the same manner as described. The night before the second surgery, the animals received 20,000 IU penicillin and 1.0 g streptomycin/10 kg body weight intramuscularly. Each dose provided antibiotic coverage for 4 days. Another dose was injected 4 days later, totaling 8 days of antibiotic coverage. This combination provides broad-spectrum coverage and is commonly used to treat infections in small animals[18] since it has a systemic and local effect on the control of periodontal infection.

Full-thickness flaps were elevated on the experimental and control sides in the area of the first to fourth mandibular premolars. The teeth were sectioned in a buccolingual direction at the bifurcation, so that the roots could be individually extracted without damaging the bony walls. After extraction, the sites were meticulously debrided, following a protocol previously described,[21] and curettage of the alveoli was performed to remove all soft tissue tags and to stimulate resorption of the cortical lining, so as to expose the marrow cavities.

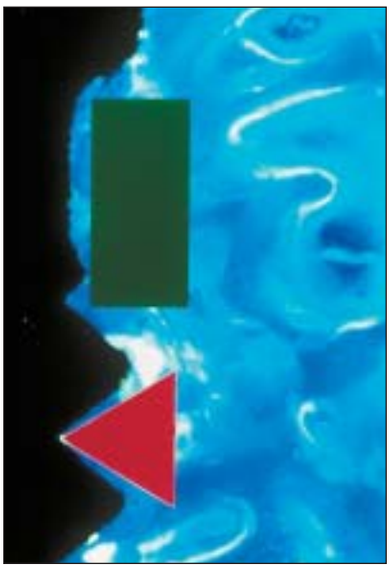

Fig 1 Areas of analysis of markers. The threaded area is represented by the red triangle between the first and second threads, and the nonthreaded area is represented by the green rectangle at the neck of the implant.

The sites were then irrigated with a 50 mg/mL solution of tetracycline hydrochloride, and Frialit-2 implants (Friadent, Mannhein, Germany) with a grit-blasted/acid-etched surface, 4.5 mm in diameter and 8 mm in length, were placed immediately, 4 implants each side, totaling 40 implants in the experiment. As described in a previous study,[19] the implants were placed according to the manufacturer's instructions, so that the top of the implant was at the bone level of the crest. The flaps were sutured with resorbable sutures. The implants used are well suited for immediate implantation since they are root-analog in shape: While they are wider cervically, adapting clinically to the alveolar walls and leaving no horizontal defects, they taper apically so that minimal amounts of bone need to be removed during site preparation. The animals were maintained on a soft diet for 14 days. Healing was evaluated periodically and the teeth were cleaned monthly with ultrasonic points. During this period, fluorescent bone markers were used to determine the sequence of bone remodeling.

Fluorochrome Bone Markers

The animals remained under observation for at least 3 months, during which 3 fluorescent markers were administered in sequence according to Cho and associates[22] to observe the rate and extent of new bone formation:

- Day 3: Fluorochrome 1: Oxytetracycline hydrochloride (Sigma Chemical, St Louis, MO), 20 mg/kg body weight intravenously
- Week 4: Fluorochrome 2: Calcein green (Sigma Chemical), 20 mg/kg body weight intravenously
- Week 8: Fluorochrome 3: Oxytetracycline hydrochloride 20 mg/kg body weight intravenously
- Three days before sacrifice: Fluorochrome 4: Alizarin red S (Sigma Chemical), 20 mg/kg body weight intravenously

All markers were prepared immediately before use with either 2% sodium bicarbonate, sterile water, or saline. Following preparation, the pH was adjusted to 7.4 and the solution was sterilized through a 0.44-µm disc filter (Schleider & Schuell, Dassel, Germany). Each animal received a total dose volume of 3 mL of each marker.

The animals were sacrificed with an overdose of thiopental 12 weeks after implant placement. Hemimandibles were removed, dissected, and fixed in 4% phosphate-buffered formalin, pH 7, for 48 hours, and transferred to a solution of 70% ethanol until processing. The specimens were dehydrated in increasing concentrations of alcohol up to 100%, infiltrated, embedded in resin (LR White; London Resin, Berkshire, England), and hard-sectioned using the technique described by Donath and Breuner.[23]

One longitudinal histologic mesial-distal section from each implant was evaluated using a fluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany) with appropriate excitation and barrier filter combinations. Color photomicrographs were taken at 125× magnification. The images from the middle third of the implants were scanned and analyzed with morphometry software (MetaMorph; Universal Imaging, West Chester, PA). The middle thirds of the implants were the only areas measured because crestal resorption is a common finding in animals, especially dogs, and also because in the mandible the apical thirds are usually close to or may even penetrate the superior wall of the inferior alveolar canal.[19]

Two distinct areas were determined for histomorphometric analysis (Fig 1). The red triangle delimits an area of 9,376 pixels between the first and second threads, revealing new bone in the middle third of the implant. The green rectangle delimits an area of 47,565 pixels adjacent to the neck of the implant where there is new bone formation on the nonthreaded surface of the implant. The sum of these 2 areas was 56,941 pixels, which comprised the mineralized tissue that formed with the preparation of the implant site and the old bone that underwent remodeling.

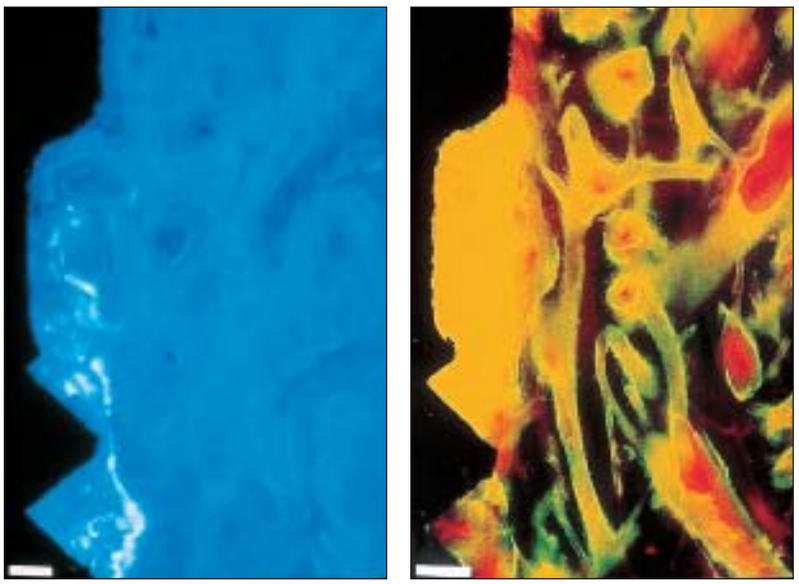

Fig 2a *(Left)* The oxytetracycline hydrochloride marker, applied 3 days after immediate implant placement, is presented in white. Bar = 140 µm.

Fig 2b *(Right)* Green represents remodeling at 4 weeks after surgery, yellow is the new bone formation at 8 weeks, and red shows new bone at 12 weeks. Bar = 140 µm.

The formation of new bone was evaluated histomorphometrically by the quantification of the bone markers, which represented the different healing periods. A certain tone of marker uptake was selected for quantification, which was then read by the computerized system.

Statistical Analysis

The Mann-Whitney test was used for comparison of the differences between the experimental/control and the nonthreaded/threaded areas. Moreover, this statistical test was used to compare the markers within groups and between groups. The level of significance was set at 5%.

RESULTS

Histologic Findings

Round or oval bands were deposited randomly throughout the previously existing bone. These fluorescent bands were irregular in form and thickness, suggesting newly formed bone. Two distinct areas were delineated as representative of the total bone surrounding the implant. According to the mean percentages of fluorescence, the mineralization and bone remodeling were not predominant in the total bone, but their linear or unicentric circular deposition was observed. This finding is in agreement with that observed by light microscopy, ie, the total amount of bone analyzed included the lamellar bone existing before implantation (old bone), along with newly formed bone adjacent to the implant and medullary canals. Distinct white, red, green, and yellow bands were observed on the periphery of the medullary spaces, not associated with them but with the mineralized tissue.

Histomorphometric Assessment

The sequential intravenous administration of fluorochrome stains demonstrated new bone formation and remodeling around the implants. Two different filters were used for fluorescence microscopic analysis: The first was more specific, comprising a narrow-band wavelength for excitation and emission of fluorescence, and the second filter presented a wider band. The tetracycline applied after 3 days could be seen separately through the first filter, with its color altered to white with a blue background (Fig 2a). The second filter comprised the other 3 colors in a single photomicrograph (Fig 2b), in which the markers calcein (green), tetracycline (yellow), and alizarin (red) could be seen. The 2 sets of photomicrographs, which represented all stains used in this study, were then digitized and the histomorphometric analysis was done.

With the second photomicrograph, it was possible to distinguish each of the 3 colors by separating them into 3 new photomicrographs (Figs 3a to 3c show specimens selected for having the most marker uptake) and calculating the percentages of new bone relative to the time of application using MetaMorph software. The mineral apposition rate could not be determined, because a continuous random mineralization was observed, without the formation of distinct bone lamellae; in addition, the observation periods overlapped.

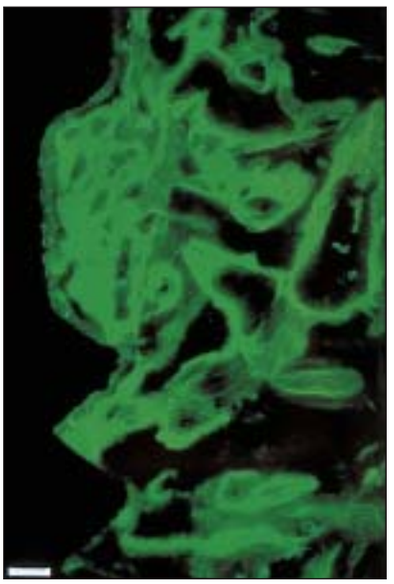

Fig 3a Calcein green marker, applied 1 month after immediate implantation. Bar = 140 µm.

Fig 3b Oxytetracycline marker, applied 8 weeks after immediate implantation. The yellow color represents the remodeling at this time. Bar = 140 µm.

Fig 3c Alizarin red marker in new bone, which was applied 3 days before sacrifice of the dogs. Bar = 140 µm.

Table 1 Percentage of New Bone Formation and Remodeling Present in Threaded and Nonthreaded Areas of Control and Experimental Sites

	Experimental group		Control group	
Time	Threaded area	Nonthreaded area	Threaded area	Nonthreaded area
3 days	15.0 ± 14.5	3.7 ± 4.2*	18.3 ± 21.2	9.0 ± 13.3
4 weeks	33.6 ± 22.8	24.4 ± 10.2	35.9 ± 21.2	34.6 ± 16.1
8 weeks	27.3 ± 26.0	15.9 ± 8.5	33.6 ± 19.5	31.0 ± 17.8**
12 weeks	53.3 ± 29.6	50.7 ± 24.4	45.8 ± 21.1	45.8 ± 23.8

Data are reported as mean percentage ± SD (Mann-Whitney test).
*There was a statistically significant difference within the group ($P = .002$).
**There was a statistically significant difference between groups ($P = .017$).

When the nonthreaded and threaded areas in the experimental group were evaluated separately, the following data were found. The mean remodeling for the nonthreaded area was 3.7% on day 3, 24.4% at 4 weeks, 15.9% at 8 weeks, and 50.7% at 12 weeks. The mean remodeling for the threaded area was 15% on day 3, 33.6% at 4 weeks, 27.3% at 8 weeks, and 53.3% at 12 weeks. A statistically significant difference was found between areas in the experimental group only for day 3 (Table 1).

In the control group, the mean remodeling for the nonthreaded area was 9% at day 3, 34.6% at 4 weeks, 31% at 8 weeks, and 45.8% at 12 weeks. The mean remodeling for the threaded area was 18.3% at day 3, 35.9% at 4 weeks, 33.6% at 8 weeks, and 45.8% at 12 weeks; there was no statistically significant difference between areas in the control group (Table 1).

The same results were found when the nonthreaded areas of the control and experimental groups were compared. However, in the 8-week observation period a statistically significant difference was found between the groups (15.9% in the experimental group and 31% in the control group) (Table 1).

When the threaded and nonthreaded areas were combined and this sum was analyzed between groups, the remodeling in both groups had similar characteristics in the degree of bone formation. Since the fluorochromes provided contrasting colors, it was possible to differentiate between periods, permitting the 3 markers to be evaluated and the changes occurring in the different phases of healing to be analyzed during the experimental period. This was possible because there was a distinct difference

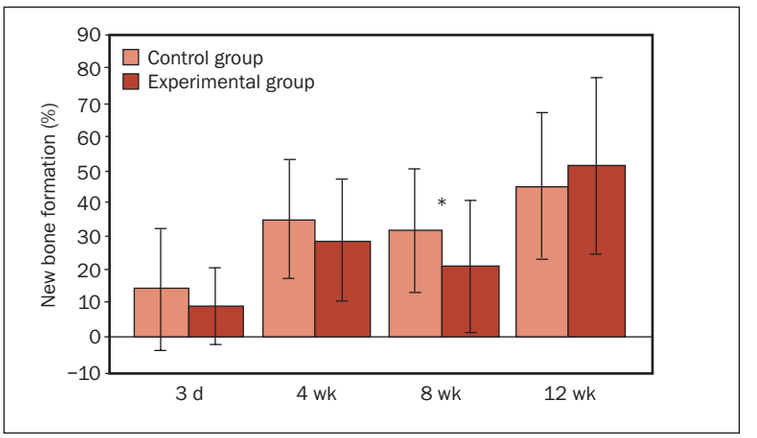

Fig 4 Percentage of new bone formation in the experimental and control groups. *Significant difference between groups at 8 weeks only (Mann-Whitney test, $P < .05$).

between new and original bone, observed by morphologic characteristics and the areas of fluorochrome marker uptake. In the experimental group, the mean remodeling on day 3 was 9%, at 4 weeks it was 29%, at 8 weeks it was 21.6%, and at 12 weeks it was 52%. In the control group, the mean remodeling percentages were: day 3, 14%; 4 weeks, 35.9%; 8 weeks, 32.3%; and 12 weeks, 45.8%. A comparison of the results of the control and experimental groups (Fig 4) in terms of labeling (periods) revealed a statistically significant difference only at 8 weeks. However, there was a numeric difference, although statistically nonsignificant, in the remodeling at day 3. The lack of statistical significance at 3 days could be explained by the low power of the test (0.2255), or there may truly have been no difference between control and test groups.

DISCUSSION

Fluorescence microscopy is a valuable research tool for evaluating the development of the interface and bone remodeling around the implants.[12] Histologic evaluation with fluorescent light shows the quality of healing and the time of new bone formation, which can be used to compare different implant surfaces[24–27] and also to evaluate regeneration of defects adjacent to the implants.[28–30] However, it is not used to quantify the formation of new bone around implants.

In dogs, periodontitis-associated plaque is easily induced,[31,32] and a pronounced increase in gingival exudation; rapid formation of periodontal pockets, attachment loss, and alveolar bone resorption; and an apical displacement of the gingival margin can be observed.[33,34] In the present study, a combination of surgically created bone defects and silk ligatures, in accordance with Schliephake and Kracht,[20] was used and resulted in Class III furcation lesions, observed both clinically and radiographically.[19] The use of fluorochromes in the histomorphometric analysis can be beneficial to evaluate whether the bone-implant contact or bone formation is affected by chronic infection.

The use of fluorochromes allows the evaluation of 2 basic variables in bone formation: *(1)* the level of cellular mineralization, which is represented by the mean distance between the parallel fluorescent markers, divided by the time between doses[35]; and *(2)* the level of bone calcification or linear extension of mineralization,[36] which indicates the extent of bone surface involved in the process of mineralization as represented by the percentage of bone containing the fluorescent marker. This second variable was used in this study to determine the possible interference of periodontal infection in immediate implantation.

Fluorescent bone markers have been used to show new bone formation,[37] with tetracycline the most frequently used marker.[38] Milch and coworkers[9,10] reported finding the fluorescent compound in areas of new bone proliferation after the administration of tetracycline as an antibiotic. They reported that tetracycline is fixed in the bone in the process of mineralization by its linkage to calcium, and once adhered, tetracycline apparently remains until the marked bone is substituted during physiologic remodeling.

Sequential polyfluorochromic marking has been used to determine when mineralization occurs.[3,39,40] The current study analyzed bone remodeling in the areas next to the interface of bone/immediately placed implants. The markers tetracycline, calcein, and alizarin demonstrated different colors and provided sequential information, and their contrast made it possible to evaluate the changes during the experimental period. These markers are fixed to calcium ions by chelating action, having the same effect,[41] and can be used comparatively.

Anneroth and coworkers[42] reported the histologic observation of tissue healing and simultaneous osseointegration of immediate implants in alveoli. After 7 weeks, there was initial healing of the implant in alveolar bone. Granulation tissue, developed from the blood clot subsequent to dental extraction, was substituted by pronounced formation of osteoids and immature bone tissue. After 12 weeks, healing of the titanium implants was complete, reorganization of tissues in the area of the implant had occurred, and the previously immature bone tissue had remodeled and been substituted by more mature bone. These observations are in agreement with the process of bone healing described by Soballe,[24] in which there was an inflammatory phase for a few days, a reparative phase after 2 weeks, and remodeling about 4 weeks after surgery. In the present study, remodeling was seen at all time periods.

There was a delay in healing during the first 3 days in the experimental group and smaller indices at 4 and 8 weeks, in contrast to the control group, in which healing was uniform. However, at 12 weeks, the experimental group showed slightly more bone formation than the control group. This initial delay in bone formation in the experimental group may have been the result of the periodontal infection, although the tissue was able to reorganize, and at the end of 12 weeks the 2 groups were similar. Based on these findings, it can be suggested that improved grit-blasted/acid-etched surfaces or perhaps different surfaces may be developed, which by better response may eliminate the influence of chronic infection in the early stages of wound healing.

According to the results shown in Fig 4, small amounts of new bone formed between implantation and 3 days of healing. This formation increased significantly between day 3 and 4 weeks, when the second marker was applied; however, this effect was not maintained between the fourth and eighth week, during which there was a decrease in bone activity in the experimental group and stabilization in the control group. There was increased activity between 8 and 12 weeks, at which time new bone formation reached its peak.

CONCLUSIONS

The results of this study support the finding that immediate implantation in the presence of chronic periodontal disease can be successful, with results similar to nondiseased sites.[19] A slight delay in healing was observed in the initial periods of observation, but at 12 weeks the difference disappeared and the results were similar for control and experimental sites. Thus, it may be concluded that experimental periodontitis was not a contraindication for immediate implantation in this model.

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