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EFFECT OF IRRADIATION ON EARLY ENZYMATIC CHANGES IN HEALING MANDIBULAR PERIOSTEUM AND BONE

A histochemical study on rats

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Abstract

The influence of irradiation was studied histochemically in healing mandibular periosteum and bone. After a cut line had been made on both sides of the mandible the rats were exposed to roentgen ray irradiation. The single doses were 15, 20, 30, 35 or 40 Gy. The animals were killed 1, 2, 4, 8, 10, 12, 16 and 24 hours after irradiation, for histochemical analysis. All enzymes, acid phosphatase, cytochrome oxidase, lactate, isocitrate, glucose-6-phosphatase and succinate dehydrogenase, showed a greater increase in enzyme staining in the irradiated cut lines than in the non-irradiated control lines. The intensity of the staining increased with time and dose over 24 hours. The observation time included an inflammatory phase with vascular, enzymatic and cellular responses to periosteal and bone injury. The increase in staining was dependent on the time after surgical trauma and radiation dose. The increase in enzyme staining probably represents the initial cell damage after irradiation.

Key words: Radiation biology; rats, bone healing, enzyme activity.

There is histochemical evidence that the activity of several enzymes increases in healing wounds (7, 8, 17, 19) and fractures (4, 15, 22, 28) during the very first postoperative hours. Few reports have been published on the distribution of the enzymes of bone in the immediate postirradiation period. Histologic radiation damage is evidenced by partial or complete destruction of cells. Histologic cell damage is, however, a relatively late phenomenon. On the other hand, enzyme histochemistry permits detection and localization of early functional changes. It is possible to show signs of cellular damage and enzyme release from lysosomes, with doses as low as 1 Gy and as early as 2 to 4 hours after irradiation (29). The radiation damage is accompanied by a decrease and later by a loss of enzyme activity (6, 14, 21). The damage caused by ionizing radiation seems to be directly related to the concentration of oxygen dissolved around the jaw bone cells at the time of irradiation (12). The level of hypoxia in irradiated bones is directly proportional to the roentgen dosage needed to result in the same level of damage (1, 5).

The purpose of the present investigation was to compare primary enzyme reactions to experimental injury in irradiated and non-irradiated rat mandibular bone and periosteum by enzyme histochemical methods.

Material and Methods

The material consisted of 40 adult albino rats of both sexes weighing 225 to 346 g.

Operative procedure. The rats were anesthetized with an intramuscular neuroleptic analgesic (fluonisone and phentanylcitrate, Hypnorm, Philips-Duphar B.V., Amsterdam, Holland), 4 mg/kg. The skin under the jaw was exposed from the external midline. The body of the mandible was exposed on both sides and 2 mm × 2 mm holes were cut in the bone with a dental drill (Fig. 1) in such a way that the cut line consisted of the periosteum and the cortical bone of the mandible. The skin was closed with Dexon sutures.

Irradiation procedure. The rats were exposed to irradiation within fifteen minutes after operation. The single doses were 15, 20, 30, 35 or 40 Gy. The physical irradiation factors were: 100 kV and 8 mA with 1.7 mm Al filtration. The tube distance was 25 mm and the field 1

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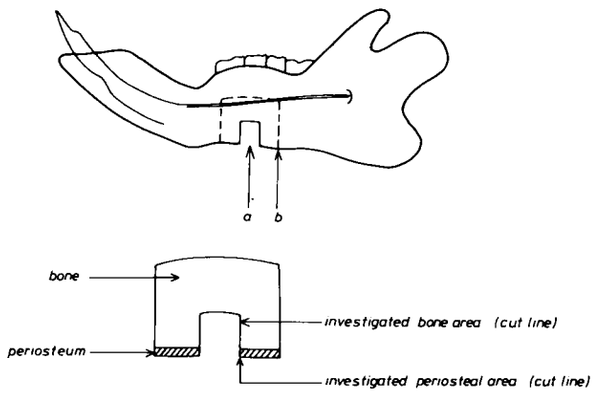

Fig. 1. Schematic drawing showing buccal view of rat mandible. Top: Operative area. a) A cavity in the lower part of the mandible. b) A cut line for histochemical specimen. Bottom: A removed part of the mandible.

cm^2. The irradiation was directed to the left side of the mandible, and the right side of the mandible was shielded with a lead plate of 2.5 mm thickness. The right side thus served as the control.

The rats were killed 1, 2, 4, 8, 10, 12, 16 and 24 h after irradiation, for histochemical analysis. All enzymes of irradiated and non-irradiated specimens were studied in each data point.

Histochemical analysis. The affected parts of the mandible were removed immediately after killing and most of the soft tissue was removed from the bones (Fig. 1). The unfixed mandibles were demineralized for 84 h in 10% disodium ethylenediaminotetraacetate (EDTA) with 0.1 mol/l phosphate buffer at pH 7.2 (3). The EDTA solution with the affected bones was kept at +4°C, and slowly circulated by a magnetic stirrer. The demineralized mandibles were frozen in a cube of dry ice attached to a chuck and cut at 14 μm in a cryostat. The frozen sections were placed on coverslips. The sections were then treated by the following histochemical techniques:

Method 1. Acid phosphatase activity was demonstrated with the azo-dye method of GROGG & PEARSE (13), as described by PEARSE (16).

Method 2. Cytochrome oxidase was studied by the method of BURSTONE (5), using 1-hydroxy-2-naphtoic acid as coupling complement.

Method 3. The following dehydrogenases were determined by the techniques described by GOHEN (11): Lactate (method 3 a), isocitrate (NADP-dependent) (method 3 b), glucose-6-phosphate (method 3 c), and succinate dehydrogenase (method 3 d). The incubation mixture consisted of Nitro-blue tetrazolium dye as the electron acceptor, veronal buffer (pH 7.4), specific substrate for each enzyme, and NAD (nicotinamide-adenine dinucleotide, 'previously called DPN') or NADP (nicotinamide-adenine dinucleotide phosphate, 'previously called TPN'), when needed for the dehydrogenase reaction.

The incubation times were 40 min at room temperature for acid phosphatase (method 1), 2.5 h at +37°C for cytochrome oxidase (method 2), 30 min at +37°C for lactate dehydrogenase (method 3 a), 45 min at +3°C for isocitrate and glucose-6-phosphate dehydrogenases (methods 3 b and 3 c), and 2 h at +37°C for succinate dehydrogenase (method 3 d).

The enzyme reactions were stopped by fixing the incubated specimens in 10 per cent neutral formalin for 5 min at room temperature. Thereafter, the irradiated and non-irradiated (control) sections were mounted with glycerin jelly, and coded blind and randomly. After that, coded specimens studied under a light microscope and the intensity of the enzyme staining were expressed as normal (+ = unruled), increased (++ = spare ruled), or intense (+++ = dense ruled) activity.

Results

The increase in normal staining occurred earlier on irradiated sides than on controls in every section. In the injured bone the cells of the inner periosteal layer showed greatly increased activity in all investigated enzymes in the cutting lines, after irradiation (40 Gy). The cells of the investigated areas wer moderately stained for lactate and succinate dehydrogenases and for cytochrome oxidase, but acid phosphatase and isocitrate dehydrogenase reacted only weakly. The activity of glucose-6-phosphate dehydrogenase was weak in all cases. The cells of the outer periosteal layer and of the endosteum as well as mature osteocytes on the irradiated side showed a weak response to all oxidoreductases analyzed.

During one to 10 h after injury, extravasated blood and inflammatory exudate filled and surrounded the operative defect. Numerous polymorphonuclear leucocytes appeared in the exudate after four h. The cytoplasm of these cells was oxidoreductase-positive. Hardly any enzyme activity occurred in the necrotic bone at the cut ends except in some cells of certain Haversian canals. Dead bone with empty lacunae was formed at a distance of 200 to 500 μm from the cut line, separating the necrotic central zone from living bone. The enzyme staining was decreased in all cut lines. However, the enzyme activities of the periosteal tissues were almost undisturbed even in the vicinity of the cut area.

No decrease in the enzyme activities of irradiated and non-irradiated areas was noted.

Acid phosphatase. Two h after irradiation, the acid phosphatase activity of osteogenic cells in the periosteum began to increase when the radiation dose reached 30 Gy (Figs 2 a, 3). The control periosteum showed activity 4 h after irradiation. The first signs of an increase in the acid phosphatase activity in the osteogenic layer of the irradiated bone were noted 4 to 8 h after irradiation (20 Gy). The control areas showed an intensification of enzyme activity after 10 h. An intense increase in staining was

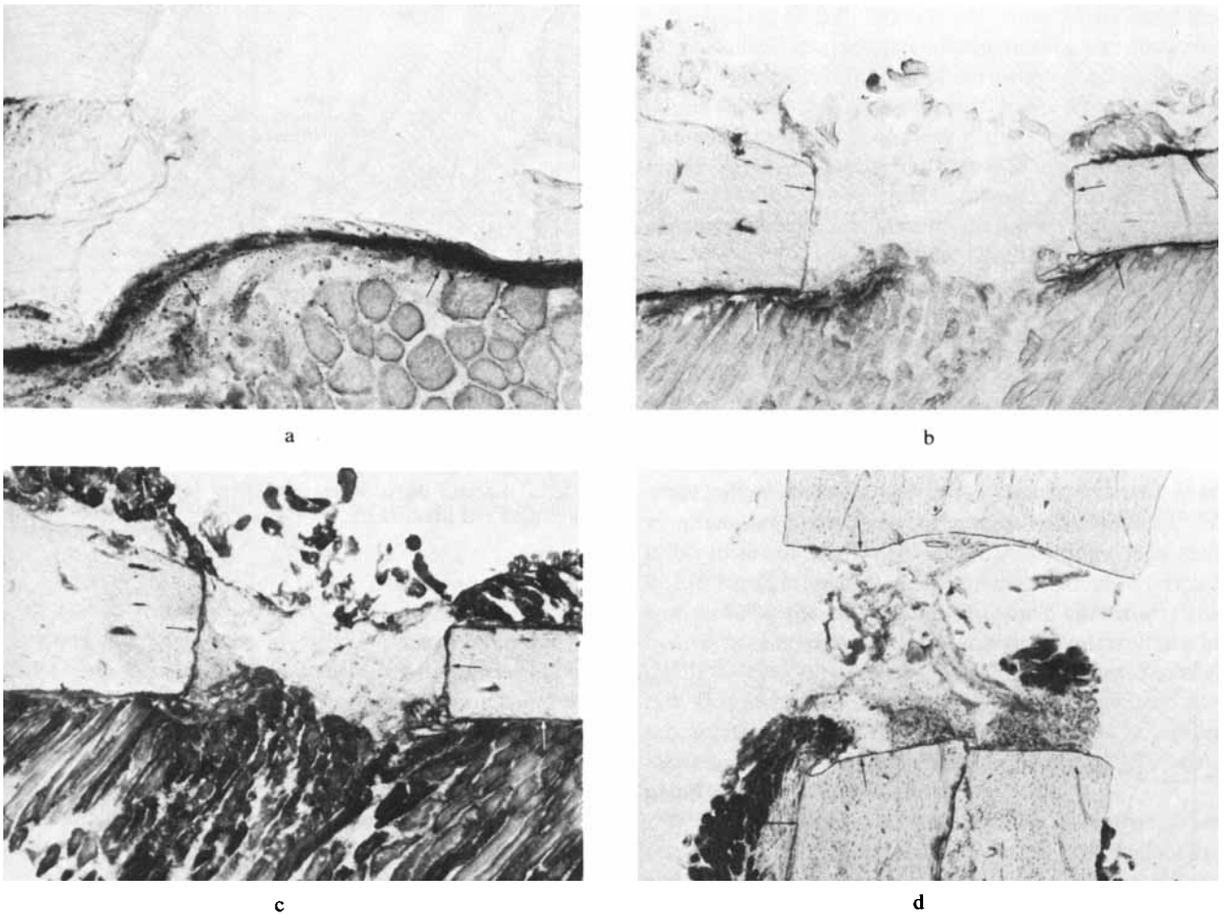

Fig. 2. Histochemical specimens from the mandible of rats. The sections are from the cut line. a) Acid phosphatase activity in periosteum 4 h after operation and irradiation with 30 Gy (×36). b) Acid phosphatase activity in periosteum and bone 8 h after operation and irradiation with 20 Gy (×36). c) Cytochrome oxidase activity in periosteum and bone 4 h after operation and irradiation with 20 Gy (×36). d) Succinate dehydrogenase activity in periosteum and bone 12 h after operation (×36).

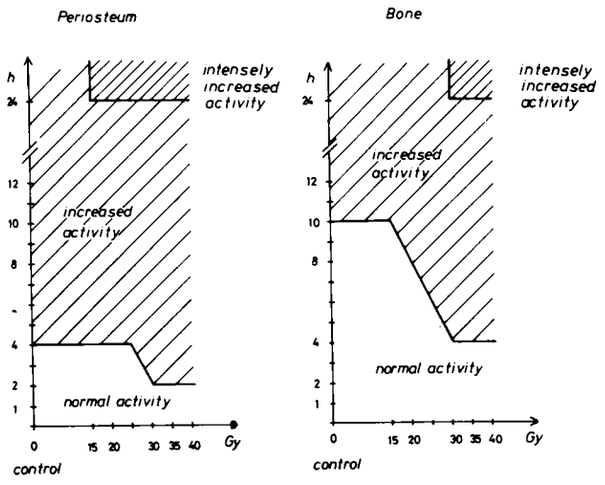

Fig. 3. Acid phosphatase activity in rat mandible after operation and irradiation.

observed 24 h after irradiation with 20 Gy in periosteum and bone, but bone required a dose of 40 Gy to achieve a similar increase.

Cytochrome oxidase. An increase in activity occurred in the cells of the inner periosteum layer. These cells were increasingly reactive to cytochrome-oxidase specific stain one to 2 h after irradiation (30 Gy). Four h after irradiation (Figs 2 c, 4) both periosteal and bone cells reacted moderately. The activity of cytochrome oxidase increased intensely in the endosteum of bone, with the periosteal reaction remaining moderate at the same dose and time. The control areas showed an increased enzyme activity in periosteum and bone after 4 to 6 h (Fig. 4).

Lactate dehydrogenase. One to 2 h after irradiation (35 Gy), lactate dehydrogenase showed beginning increased reactions in periosteal and bone cells. After 8 to 10 h the reactions were intense both in periosteum and bone. The controls showed normal activity 8 to 12 h after irradiation (Fig. 5).

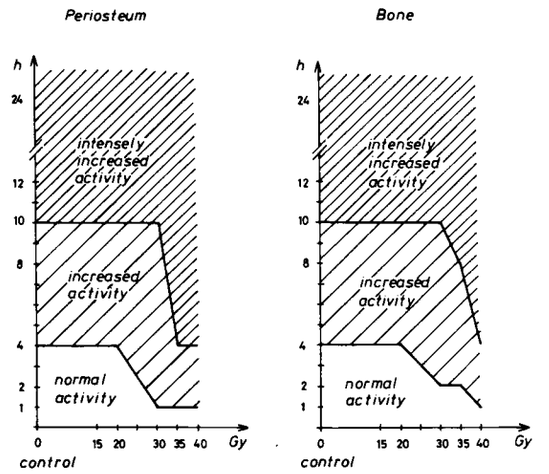

Fig. 4. Cytochrome oxidase activity in rat mandible after operation and irradiation.

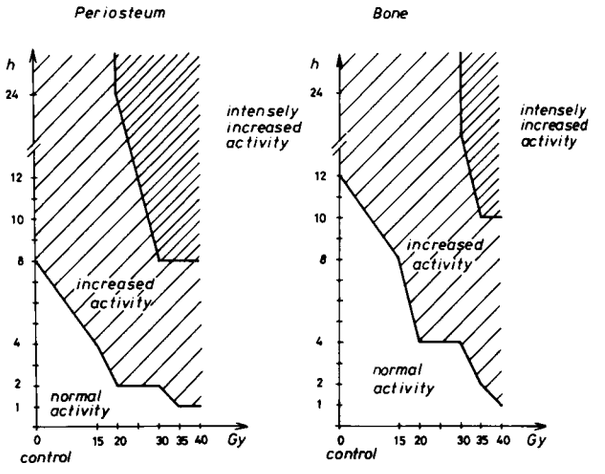

Fig. 5. Lactate dehydrogenase activity in rat mandible after operation and irradiation.

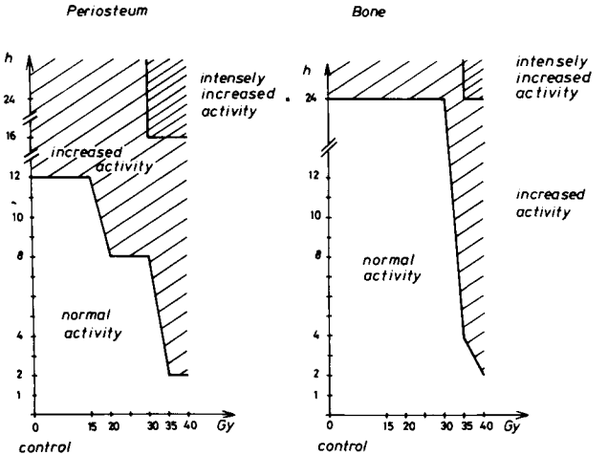

Fig. 6. Isocitrate dehydrogenase activity in rat mandible after operation and irradiation.

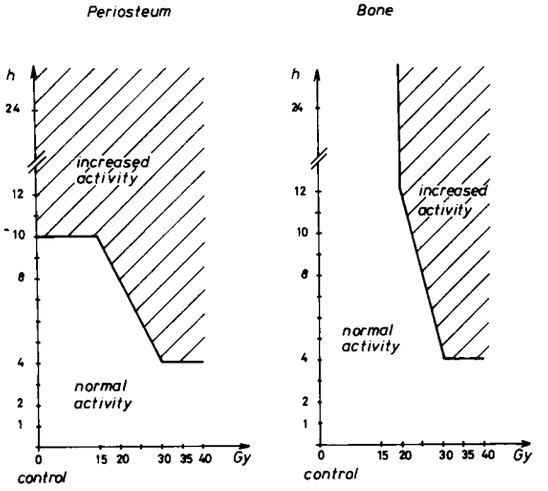

Fig. 7. Glucose-6-phosphatase dehydrogenase activity in rat mandible after operation and irradiation.

Isocitrate dehydrogenase. After a radiation dose of 35 Gy the activity of oxidoreductases increased, especially the irradiated periosteum showed increased staining after 2 h, and bone after 4 h. The staining became intensely increased after 16 to 24 h, and the cells showed an intense increase in isocitrate activity at that time. At 12 to 16 h after the bone injury in controls there was a weak increase in isocitrate in the periosteum, and at 24 h in the bone (Fig. 6).

Glucose-6-phosphate dehydrogenase. The onset of increased activity of glucose-6-phosphate dehydrogenase in the injured and irradiated bone occurred 4 h after irradiation (30 Gy). This was visible both in the cells of the inner periosteal layer and in the bone (Fig. 7). In all these cells the activity of the enzyme was very weak. No increased activity of glucose-6-phosphatase dehydrogenase was seen in the injured control bone. A slight reaction in the endosteum of bone marrow cells was identical with that of irradiated periosteal cells after 10 h (Fig. 6).

Succinate dehydrogenase. The activity of succinate dehydrogenase began to increase one to 2 h after irradiation (35 Gy) in periosteal cells, but in bone the delay was 2 h. The intensity of the reaction of irradiated bone and periosteal cells was almost identical but appeared 2 h later (Figs 2 d, 8). In the controls the first increase in the activity occurred 8 to 12 h after irradiation.

Discussion

All enzymes investigated showed an accelerated increase in enzyme activity in the irradiated cut lines as compared with the non-irradiated cut lines. The distribu-

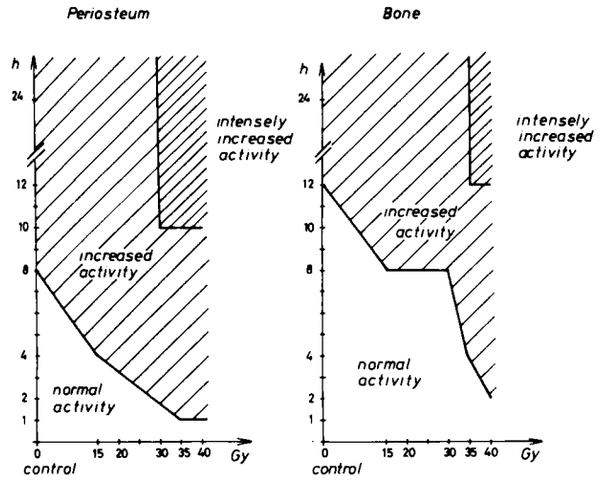

Fig. 8. Succinate dehydrogenase activity in rat mandible after operation and irradiation.

tion and increase of the investigated enzymes in the injured bone was similar to that described by other workers in fractured bone (3, 22, 23). The surgical trauma and the radiation injury studied by us were certainly not identical with a bone fracture as regards pathogenesis and healing process. Nevertheless the primary enzymatic reactions were similar and the radiation injury enhanced enzyme activity in periosteal and bone injuries. The enzyme activity increased with increasing radiation dose (between 0 and 40 Gy).

In normally growing bones, proliferation cells of either periosteal or perichondrial origin show high enzyme activity at the onset of fracture repair (19, 23). In the present investigation, a simultaneous radiation injury promoted the enzyme staining even further.

On the other hand, decrease in enzyme activity seems in general to be an early sign of necrosis (18). This was also seen in the present investigation. At a depth of 200 to 500 μm from the cut line, in the immediate vicinity of the injured area, the subperiosteal osteocytes showed a decrease in enzyme activity. The same decrease was seen in early reports (22). The area investigated was outside the immediate vicinity of the cut line (necrosis) in periosteum and bone. BACQ & ALEXANDER (2) suggested that cell damage after irradiation may be due to a disruption of cellular organization and a disturbance of the phospholipid membranes of lysosomes releasing hydrolytic enzymes, such as acid phosphatase. Liberation of hydrolytic enzymes from lysosomes would result in extensive damage of proteins, nucleic acids and other molecules in the cells (9). Irradiation to lysosomal membranes induces the release of enzymes, or, as an indirect effect, the liberation of hormones from lysosomes (24). Thyroid hormones are involved in the overall reorganization of intracellular mitochondrial and lysosomal membranes (24, 26, 27).

RAEKALLIO et coll. (20, 22, 23) showed that early fracture healing is characterized by increasing enzyme activities in the peripheral zone of periosteum and endosteum during the first few days and even hours after irradiation. The initial increase in enzyme activity occurred in osteoblasts and osteogenic cells. This is in agreement with the present results. When tritiated thymidine was used to stop cellular division, the initial proliferative response to fracture was seen at the same time (28). In this study the first signs of increased staining in the periosteum were noted at 2 h, when the radiation dose was 30 Gy, compared with 4 h in the non-irradiated controls. Irradiation and trauma, acting as stimuli, activate defense systems after a relatively short mobilization time. Thus, the initial increase in enzyme activity probably represents an adaptive defense mechanism of the local cells (17, 19).

With increasing postoperative time, the intensity in enzyme reactions increased at the same radiation dose. This is due to an enzymatic response to injury of the local cells, and to immigrating inflammatory cells, which are rich in enzymes. On the other hand, lysosomal enzymes release hydrolytic enzymes as the first sign of intracellular catabolism (19). The increase in oxidoreductases, however, is a sign of several metabolic processes associated with regeneration (23, 25). After doses of 30 to 35 Gy a strong increase occurred in the activity of all enzymes, as compared with the control side.

Destroyed cell organelles are not able to synthesize new enzymes, which leads to a decrease in cell metabolism and to cell death, later manifested by a decrease in enzyme activity. The decrease in the oxidoreductase and acid phosphatase activity of subperiosteal osteocytes in the irreversibly injured zone in the immediate vicinity of the cut line should be regarded as a sign of imminent necrosis. This is histochemically demonstrable as early as one to 2 h after injury, as a decrease in enzymes. Necrosis in this area is histologically evident 2 days after operation (21, 22).

When the radiation dose in the investigated area is high (35 Gy), the first reaction in the irradiated cut line is essentially a sterile inflammation as a consequence of surgical trauma, radiation injury and haemorrhage. The lower the pO_2 values further away from the cut line, the more likely the neogenesis of cells and blood vessels in partial anaerobiosis. The tissue pO_2 pressure is lowered to 10 to 15 mmHg (1). Owing to the scarcity of oxygen $NADH_2$ (reduced nicotinamide-adenine dinucleotide) pyruvate is reduced to lactate by lactate dehydrogenase (10). This enzyme is NAP-dependent and thus lipoamide dehydrogenase, oxidizing $NADH_2$ to NAD, could promote the reduction of pyruvate to lactate. The acidic environment, promoted by lactate, is known to characterize the early phases of healing (25).

The results of this investigation are in agreeement with earlier reports on fracture healing (20, 21, 22), in which the first signs of increase in enzyme activity were seen 4

to 12 h after injury, and correlated with enzyme activities on the control side. Irradiation injury accelerated the enzyme mediated staining first in periosteum and later in bone.

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