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Original Article

Hylonome

Effect of ifosfamide on bone healing

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Abstract

The effect of ifosfamide on bone healing was tested in a controlled experiment of fibular osteotomy in immature rabbits. Standardized shaft osteotomy was implemented in 10 experimental subjects (group 2) and 10 controls (group 1). Experimental animals received a 50 mg/kg ifosfamide dose by intraperitoneal injection on the fourth post-operative day, and for five days thereafter, while controls received injections of distilled water. After five weeks, all animals were submitted to pharmacological euthanasia and the resulting bone callus samples were studied with histomorphometry, using hematoxylin-eosin stain. Group 2 presented smaller bone volume (69.03% versus 84.98%), larger fibrosis volume (30.96% versus 15.02%), and larger resorption surface (22.02% versus 16.17%) than group 1 (all $p \leq 0.05$). We conclude that ifosfamide is able to alter the physiological bone healing process by producing a less mature callus (characterized by a smaller quantity of bone tissue), a larger quantity of fibrous tissue, and a smaller resorption surface.

Keywords: Ifosfamide, Bone Healing, Side Effect

Introduction

Surgical treatment of bone sarcoma with limb preservation is successful in 50 to 80% of attempts, and the associated disease-free survival is more than 60%[1-3]. These high success levels accompanied the advent of systemic chemotherapy with cytotoxic drugs, adequately combined in order to control distant metastases and reduce the tumor's reactive zone, allowing the preservation of the limb with or without bone substitution[1-3].

Some of the chemotherapeutic agents that have been successfully used for treating bone sarcoma probably inhibit osteogenic activity, a basic condition for incorporating bone allografts[4-6]. In clinical practice, this side effect of chemotherapeutic drugs is evidenced by the high rate of graft-associated complications leading to graft fragmentation and deep infection, including non-union, delayed or inadequate incorporation[4-6].

Ifosfamide, a cytotoxic agent, is a component of chemotherapeutic protocols used in many countries for treating osteosarcomas[7]. Although previous reports have suggested that the use of ifosfamide is related to bone alterations[8,9] there are no reports regarding the effects of this drug on the post-operative bone healing process.

We used an experimental model of bone injury in immature rabbits as previously reported[10] to evaluate morphological alterations produced by the administration of ifosfamide during the healing of injured bone tissue.

Materials and methods

This study conformed to the guiding principles of the Declaration of Helsinki involving experimental animals, and was approved by the Research Ethics Committees at the University of São Paulo and the Bahia School of Medicine and Public Health.

Animals – experimental groups

Twenty immature male albino New Zealand rabbits (age: 1.5 months; weight: 500-550 g) were assigned to the control or experimental group (ten animals each).

Animals were acclimated in the animal care facility for several days, and were housed in individual cages during the entire study period with water and chow diet *ad libitum*.

The authors have no conflict of interest.

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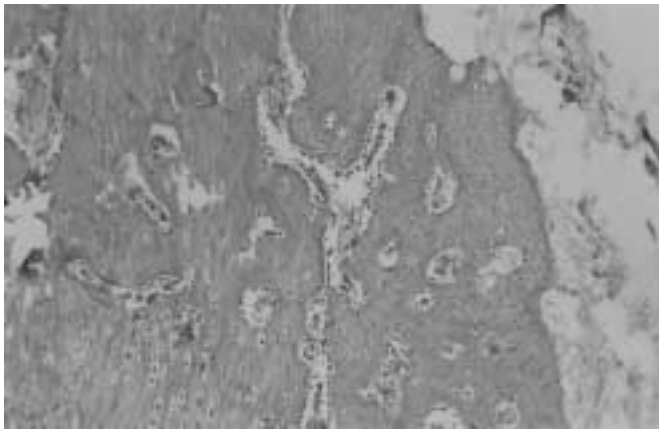

Figure 1. Callus of experimental group containing large amounts of fibrosis and smaller bone volume.

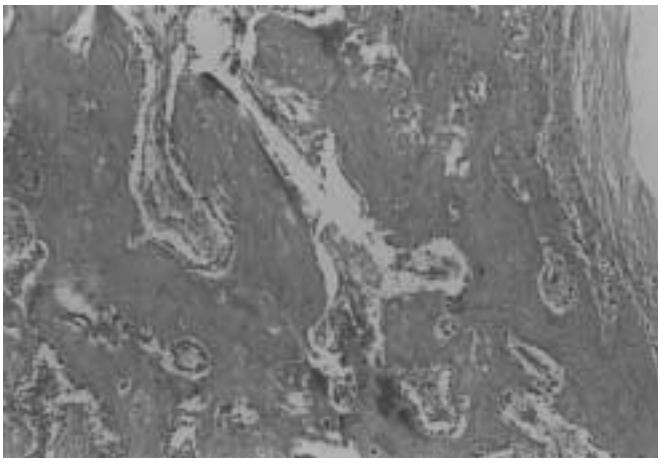

Figure 2. Callus of control group containing more bone volume and less fibrosis.

Experimental design

Food was suspended eight to ten hours prior to administration of anesthesia. To decrease the vagal tonus, each animal received 0.2 mg/kg dose of atropine sulphate by intramuscular injection. Animals were anesthetized by intraperitoneal injection of ketamine (25.0-30.0 mg/kg of body weight) and intramuscular injection of diazepam (5.0 to 10.0 mg/kg of body weight).

This experimental model of osteotomy in rabbits was reported in 2001[10]. Under an aseptics conditions technique, the fibula of each animal was accessed by a lateral incision of approximately 5 mm on the right pelvic limb. After division of the skin and subcutaneous tissue, the fascia of fibular muscles and periosteum were opened and dissected from the cranial portion of the fibula. Shaft osteotomy was performed on the cranial portion of the exposed fibula, using an electric saw with a standardized blade (10.0 mm wide and 0.5 mm thick). The incision was closed by layers, using absorbable 5-0 polyvycril sutures for the fascia and 5-0 mononylon sutures for the skin.

In the experimental group, (group 2), a total dose of 50 mg/kg of ifosfamide was divided and administered via intraperitoneal injection on the fourth post-operative day, and for five consecutive days thereafter. In the control group, the same volume of bi-distilled water was administered under similar conditions.

After five weeks, animals of both groups were anesthetized and killed with a 2 ml intracardiac injection of potassium chloride. The fibula of each animal was removed, dissected from the surrounding soft tissue, and fixed in 10% formalin for microscopic evaluation. Formalin-fixed bones were decalcified with 7.5% nitric acid, embedded in paraffin and longitudinally sectioned. Histological sections (7 μm thick) were stained with hematoxylin and eosin stain prior to optical microscopic examination.

Histomorphometric evaluation of the callus

Three histological sections were analyzed for each animal. After the cuts had been chosen, a preliminary analysis was performed at 100X magnification in order to define the area of the callus, defined by the regions associated with significant periosteal thickening, i.e., the area where the cortical bone thickness was more than doubled. Histomorphometric evaluations of all microscopic fields were performed using a test eyepiece reticule with 10 parallel lines and 100 points containing a grid with a total area of 10,500 μm^2 (Zeiss 23-9901) at a magnification of 200X. The associated parameters analyzed were bone volume, fibrous volume, bone formation surfaces and bone resorption surfaces, taken from a previous report by Parfitt[12] and Compston[11].

Statistical analysis

Results are reported as mean±standard deviation. Differences between the groups were assessed using the Mann-Whitney test for independent non-parametric data and the level of significance was $p<0.05$.

Results

All animals survived to the end of the study. Neither wound infection nor dehiscence was observed in the animals of either group. However, intense fur waste (a known consequence of treatment with chemotherapeutic agents such as ifosfamide) was observed in the experimental group animals.

Macroscopic and microscopic evaluations

Macroscopic and microscopic evaluations demonstrated that all osteotomies were consolidated by the end of the study. How-

ever, microscopic analysis revealed that animals in the experimental group had less bone tissue, more fibrous tissue (Figures 1 and 2), and more tissue-bearing hondroid features in the newly formed bone. We were unable to quantify "cartilaginous tissue" since hematoxylin-eosin staining is not adequate for this task.

Bone and fibrous volume observed in both groups are presented in Tables 1 and 2. The histomorphometric parameters for measuring formation surfaces and resorption surfaces in the control and experimental groups are presented in Tables 3 and 4. Ifosfamide appeared to promote a decrease in the resorption surface but not in the formation surface.

Group	Bone Volume
Control	84.98 (±9.78)
Experimental	69.03 (±20.02)*
*p<0.05 (Mann-Whitney test)	

Table 1. Histomorphometric data of bone volume (%).

Group	Fibrous Volume (%)
Control	15.02±9.78
Experimental	30.96±20.03*
*p<0.05 (Mann-Whitney test)	

Table 2. Histomorphometric data of fibrous volume.

Group	Formation Surface	Active Formation Surface
Control	41.7±4.12	31.99±4.28
Experimental	44.82±4.36*	34.34±5.54*
*p>0.05 (Mann-Whitney test)		

Table 3. Histomorphometric description of bone formation surface measurements (%).

Group	Resorption Surface	Active Resorption Surface
Control	22.02±4.96	3.28±2.18
Experimental	16.17±6.41*	4.57 (±2.30)
*p<0,05 (Mann-Whitney test)		

Table 4. Histomorphometric description of bone resorption surface measurements (%).

Discussion

Ifosfamide is a non-selective anti-neoplastic agent which affects all stages of cell cycle proliferation. Its main mechanism of action involves alkylation via nucleophylic unimolecular substitution, which produces highly reactive intermediate products that bind co-valently to several nucleophylic compounds. Among all cellular components, DNA is the most sensitive to the action of alkylating agents. This mechanism of action incurs a biochemical alteration in the DNA that inhibits and modifies its replication, which in turn inhibits both mitosis and protein synthesis, both of which are important to bone reparation[7].

Several adverse effects of anti-neoplastic drugs on the healing of bone fractures have been reported, among them pseudoarthrosis, late union, non-integration of grafts and deep infections[13]. This implies that these drugs have an inhibitory effect on both osteointegration and bone healing. However, there are no prior investigations of the effects of ifosfamide, one of the most commonly used drugs in orthopaedic oncology.

The rabbit was chosen as the animal model because it is widely used in studies of bone reparation[10,14]. A high incidence of primary malignant bone tumors in childhood and adolescents has been observed in clinical practice; as a result, immature animals were used to investigate the role of ifosfamide on bone repair in developing skeletons[10].

A 50 mg/kg ifosfamide dose was administered by the intraperitoneal route, according to the recommendations for human bone cancer treatment described in a Brazilian protocol[13,15].

The tissue type found in the reparative callus of the experimental animals was different from the control animals. Ifosfamide appeared to increase fibrosis volume and decrease bone volume. These findings deserve further comment. First of all, the decreased bone volume observed in experimental animals provides clear evidence that the formation of both the internal and external calluses were inhibited. Secondly, the increased fibrous volume observed in the experimental animals suggests the presence of a more immature callus, since the initial phases of the bone-repair process is characterized by large amounts of fibrosis. Also, the finding that isles bearing chondroid features were less frequent in the control group suggest that the cartilage-substitution stage was less developed in the experimental animals.

The formation of bone callus containing large amounts of connective tissue has been observed in previous experiments[4,7]. In addition, other chemotherapeutic drugs (e.g., methotrexate, doxorubicin) have been shown to inhibit bone formation in experimental fractures and during incorporation of bone allografts[4,7].

The inhibition of protein synthesis may be responsible for the negative effect of ifosfamide on the formation of bone tissue in the callus. Biochemical DNA alteration produced by drugs could lead to the inhibition of RNA and protein synthesis and the subsequent reduction of osteoid tissue synthesis. Other potential mechanisms could involve a direct influence on other physiological phenomena involved in bone reparation, such as vascular neo-formation and inhibi-

tion of osteoprogenitor cells. Nonetheless, these questions require proper studies for their elucidation.

The callus of the experimental group presented with a larger formation surface and smaller resorption surface (Tables 3 and 4). Intense bone formation occurs in the early stages of repair and resorption increases in the final stages. It is possible that under the action of ifosfamide the production of woven bone could be delayed and therefore the amount of bone volume was smaller but the bone formation surface presented a trend to growth in the fourth week (late stage). This reinforces the hypothesis of the callus in the experimental group being at a less advanced stage of maturation than the callus in the control group.

Ifosfamide produced a significant decrease in resorption surface; however, the active resorption surface (e.g., the one covered by osteoclasts) presented a trend to growth, although this trend did not reach statistical significance at the 0.05 level. This suggests that the callus produced in the experimental group had a smaller resorptive surface, but presented more osteoclasts.

The results of the present study agree with those from previous experiments with chemotherapeutic drugs. Friedlaender et al.[7] showed that doxorubicin inhibited bone resorption more than methotrexate. Neither of these two drugs altered the percentage of trabecular bone surface covered by osteoclasts, but a trend to an increased number of osteoclasts per unit of trabecular perimeter was noted, as well as a significant increase in the percentage of trabecular surface occupied by Howship's lacunae (where no osteoclasts are found). In addition, the study was unable to explain how the agents used to inhibit bone resorption instead increased the number of osteoclasts. Another study of bone grafts verified that methotrexate increased osteoclastic activity and inhibited interior osteoclast activity without affecting the periphery of the bone tissue[4]. In this work, the effect of adriamycin was not clarified, and was thus attributed to unknown biological factors or to the drug's effect upon endothelial cells and graft re-vascularization.

The effect of chemotherapeutic drugs on bone resorption has not been fully clarified. Here, ifosfamide produced either a decrease in resorption or activity alteration, but the same did not apply to the number of osteoclasts. This effect could also be attributed to the delay at the beginning of the resorptive stage of the callus or the inhibition of the angiogenic stage. These questions, however, must be addressed in other investigations.

In conclusion, we demonstrate that ifosfamide altered the process of bone healing in a rabbit model of osteotomy. This effect is characterized by the production of a callus at a earlier reparation phase than in the control group. The experimental animals presented a significantly larger fibrosis volume and a relatively smaller bone volume inside the formed callus. Bone callus formed under the effect of ifosfamide presented a significantly smaller amount of bone resorption surface and a tendency towards an increase in active resorption surface. These results should be considered when treating children with primary malignant bone tumors.

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