Original Article

Evaluating the effectiveness of gel formulation of irradiated seed lectin *Cratylia mollis* during bone repair in rats

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Abstract

Context: Regeneration corresponds to the replacement of damaged cells with ones that have the same morphology and function. For experimental evaluation of materials that may favor the process of bone healing, defects are created with dimensions that prevent spontaneous regeneration. For the development and use of new drugs, it is necessary to study its effects in vitro, which depends on the formulation, concentration, and rate of irradiation in vivo and the route and frequency of administration; thus, it is possible to characterize the physiological and molecular mechanisms involved in the response and cellular effects. **Objective:** The objective of this study was to assess the effect of Cramoll-1,4 on the process of bone repair. **Materials and Methods:** A formulation of biopharmaceutical lectin Cramoll-1,4 at a concentration of 300 mg/100 mL was applied in a single application via gamma radiation and its effect on the process of bone repair in rats was assessed. **Results:** Histologically, it was observed that the bone defect is coated by loose connective tissue rich in fibroblasts, providing a range similar to the thick bone original and competing with site of new bone formation. This prevented direct contact between the formulation and experimental bone tissue, as, despite its proven effectiveness in experiments on the repair of skin lesions, the formulation used did not promote bone stimulation that would have promoted the tissue repair process. **Conclusion:** Because of the direct interference of loose tissue repair that prevented direct contact of the implant with the bone interface, the formulation did not promote bone stimulation.

Key words: Bone repair, cramoll-1,4, natural products, irradiated gel

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Introduction

Presently, the trauma has been seeking new methods for more efficient bone repair. Hence, many researches have been developed to improve the bone-healing process; these studies involve not only surgical technique but also the use of biological resources and molecular techiniques.[1]

The bone repair process involves a complex cascade of biological responses, is affected by local and external factors, and is regulated by the interaction of different mechanisms. Increase or decrease in bone-repair capacity is directly related to changes in remodeling.[2]

Bone regeneration is understood to be the replacement of damaged cells by other morphologically and functionally identical cells. Bone lesions with small dimensions are repaired easily, while fibrous scar, as a peculiar characteristic of this type of fabric, needs a repair mechanism similar to that of embryonic osteogenesis.[3] However, the regenerative capacity of bone tissue is limited by the extent of the injury,

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in this sense, extensive bone defects caused by trauma, tumors, infections, and developmental abnormalities do not regenerate spontaneously, posing a challenge to the scientific community.[4]

In any experimental study, a critical size defects (CSD) should be established, which is defined as the smallest intraosseous defect that will not heal spontaneously throughout the animal's life, affecting the formation of fibrous connective tissue rather than bone formation.[5] In rodents, lesions with diameter >2.7 mm are considered CSD.[6]

Lectins are a structurally heterogeneous group of proteins, glycosylated or not, non-immune origin that have the ability to recognize at least a reversible binding site specific monosaccharides or oligosaccharides.[7] The cell surfaces are rich in glycoproteins (glycosidic receptors) that potentially interact with lectins by carbohydrates attained portion; this lectin-cell interaction is the molecular basis that can trigger several changes in biological organisms.[8] Regarding the use of glucose-mannose lectins in the healing process, the lectins of *Canavalia brasiliensis*, *Dioclea violacea*,[9] *Canavalia ensiformis,* and *Cratylia mollis*[10,11] used in the treatment regimens proved to be effective therapeutic agents.

The present study was aimed to evaluate the effectiveness of using the gel formulation of irradiated *Cratylia mollis* lectin (isoforms 1 and 4) in experimentally induced bone defects in rats, considering the reduction of time to repair tissue and the histological characteristics of repair process in relation to experimental treatment applied, assessment of serum biochemistry, and bone mineral measurement.

Materials and Methods

Gel formulation of spent cramoll-1,4

Carbopol® was used as a vehicle suspended in boric acid buffer (pH 6.0) at 25°C. After extraction and purification, Cramoll-1,4 solution was added in sufficient quantity to achieve the final concentration of 300 μg in 100 μL of the gel. Irradiation was performed at room temperature using cobalt-60 at a dose of 15 kGy.[12]

Selection and sample size

All procedures related to the use of animals were submitted to and approved by the Ethics Committee on Animal Experimentation of Federal University of Pernambuco (Process 016036/2007-10).

We used 12 90-day-old female albino Wistar rats (*Rattus norvegicus*), weighing 300 ± 50 g, and produced two critical defects in each animal, where each rat was a control for herself, and 3 rats were euthanized at the end of each experimental period (15, 30, 45, and 60 days, postoperatively).

Induction of critical defect

The animals were pre-anesthetized with atropine sulfate at a dose of 0.04 mg/kg/intramuscularly. After 10 min of anesthesia, a combination of 10% ketamine hydrochloride (90 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) was applied intramuscularly. Trichotomy was performed in mouse calvarial and antisepsis with an alcoholic solution of 10% iodine polivilpirrolidona, followed by local anesthesia with bupivacaine and section 2000 IU of skin and subcutaneous tissue. We produced two circular bone cavities (in each parietal region) using a trephine drill of 5 mm diameter [Figure 1].

One of the cavities was filled with the experimental treatment, while the other was filled with a gel as a control excipient. Later, the two cavities were covered by an absorbable cellulose membrane (Bionext®), did not occur to the passage of material from one cavity to another. The plans were sutured surface.

In the immediate postoperative period, the animals were kept in an environment without air conditioning and subjected to heating by a light source, avoiding hypothermia, cardio-respiratory depression, and death, and taking care to protect the eyes with gauze moistened with 0.9% saline.

Also, analgesia was instituted immediately after surgery by administering Dipyrone sodium (0.15 mg/kg) intramuscularly and maintenance for 3 consecutive days, added to drinking water (40 mg/kg).

The administration of antibiotics as a prophylactic measure was not carried out. To control the environment in the operating room, aseptic use of personal protective equipment suitable for sterile surgery and antisepsis of the area to be operated. Animals were monitored daily for signs of pain (hair bristling

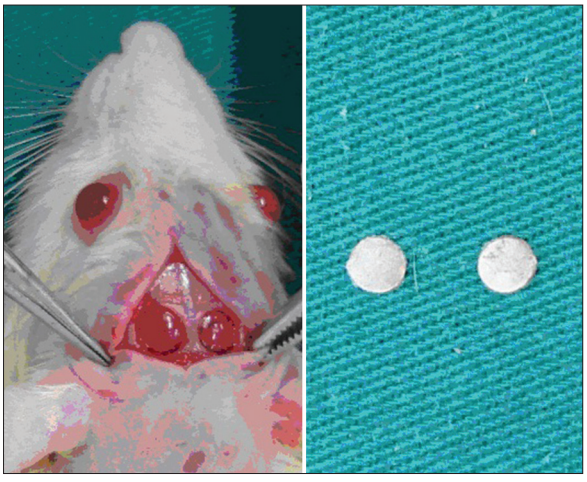

Figure 1: Critical defect: Production of two circular bone cavities (in the parietal region) measuring 5 mm in diameter

and anorexia), behavioral changes (lethargy and drowsiness), or neurological (ambulation and head deviation) damage and/or infectious. Although it is an invasive surgery, the animals showed normal behavior of the species and ingestion of food and water.

Euthanasia and specimen collection

In the pre-set from 8:00 AM, with 2 h fasting, the animals were anesthetized and 4 mL of blood was collected from each animal by cardiac puncture into sterile test tubes. The animals were then given an overdose of thiopental sodium, intraperitoneally, for completion of euthanasia.

Serum biochemistry

Calcium, magnesium, and phosphorus

The determination of calcium (Ca) levels was performed by the Arzenazo DiaSys III method, the determination of phosphorus (P) by the Mobilidato DiaSys UV method, and determination of magnesium (Mg) by the Xilidil DiaSys method.

Albumin and alkaline phosphatase

The determination of albumin (ALB) and alkaline phosphatase (ALP) was performed by using Automation Architect (Abbot Diagnostics) in blood serum.

Bone mineral dosage-proportion of Ca, P, and Mg

We reserved the left tibia of each animal, deposited individually in porcelain crucibles in an oven at a temperature of 100°C for 2 h, and determined the proportion of Ca, P, and Mg using atomic absorption spectrometry (Shimadzu AA-6300).

Collection and processing material for histological analysis of bone repair

The skullcap was removed, immersed in 10% formaldehyde solution in PBS (isotonic saline solution buffered with sodium phosphate), pH 7.2-7.4, for at least 24 h. After fixation, the caps were decalcified in a solution of 10% nitric acid for 2 h at 60°C. They were then washed in distilled water, dehydrated in increasing battery of alcohol, cleared in xylene, and embedded in paraffin. The samples were sectioned in semi-serial cuts 5-μm thick and stained with hematoxylin-eosin and Masson's trichrome.

Statistics

Statistical analysis was conducted by using Student's *t* test for independent samples, adopting a 5% statistical significance ($P < 0.05$). The software used for data analysis was STATA version 9.0.

Results and Discussion

The serum values of ALB, ALP, Ca, P and Mg were within the limits established by Mitruka and Rawnsley,[13] showing a difference related to the values of ALP (56.8-128 U/L), while, across the dosage of the trial period, ALP was high (214.0-286.0 U/L) [Table 1].

The levels of ALP [Table 1] were not different when comparing the experimental periods. On an average, highest level of ALP was found 45-days postoperatively. However, this level was higher than the established values by the Hematology Baseline and Clinical Chemistry Values for Charles River Wistar rats for females aged 19-21 weeks[14] (39-177 U/L) and (39-216 U/L) established by Olfert *et al*.,[15] which can be justified by the fact that collections were made after 15 days of induction of critical defect, which coincides with the production phase of the collagen matrix having a higher production of alkaline phosphatase.[16]

Analyzing measurements of albumin [Table 1] in four experimental periods, it was observed that there was an increase in the average group of animals sacrificed at 30 days after the induction of critical defect as compared to that in other groups, but the difference was not statistically significant. The average albumin in all experimental periods ranged between 4.1 and 4.4 g/dL, with great variability in the group with 30 days of experimentation.

At 30 days of the experiment, a mean of 10.7 mg/dL calcium was detected, and the difference was statistically significant as compared to groups with 15 days ($P = 0.050$) and 60 days of the experiment ($P = 0.048$) [Table 1].

Evaluating the behavior, there was a significant increase of calcium at 30 days post-operatively, decreasing the concentration to 60 days after the start of the intervention.

Despite the variation found (10.7 mg/dL), calcium levels were maintained within the reference values established by Mitruka and Rawnsley[13] (7.2-13 mg/dL). As the calcium concentration

Table 1: Mean±SD of serum albumin, alkaline phosphatase, calcium, phosphorus and magnesium in animal experimental periods

Experimental periods	ALB. g/dL^{ns}	F.A. U/L^{ns}	Ca^{+} mg/dL	P mg/dL^{ns}	Mg mg/dL
15 days	4,17±0,06	246,33±0,06	10,10* ±0,21	5,81±0,74	3,13** ±0,06
30 days	4,40±0,56	220,00±90,53	10,70* ±0,36	5,50±0,51	2,33** ±0,15
45 days	4,30±0,17	286,33±41,63	10,40±0,70	6,26±1,61	2,60±0,52
60 days	4,17±0,40	214,00±61,29	9,70* ±0,58	4,99±0,15	2,27** ±0,15

*Difference significant at 5% probability level of interaction between calcium and experimental period. **Significant difference at 1% probability level of interaction between magnesium and experimental period. ns: Not significant. ALB: Albumin. Ca: Calcium. P: Phosphorus. Mg: Magnesium

remained constant, only in extreme circumstances such as malnutrition or hyperparathyroidism, the serum concentration of this mineral in its ionized form is altered.[17-19]

According to Cohen and Roe,[20] 80% of plasma calcium are found bound to albumin. In this sense, the amount of calcium should always be interpreted together with the concentrations of albumin. Table 1 shows the relation of the changes in the levels of calcium and albumin in the experimental periods. Phosphorus levels did not vary between different follow-up times, averaging a concentration between 5 and 6 mg/dL in all experimental groups, being within the parameters set as normal (5.3 to 8.3 mg/dL)[21] with the 2:1 ratio of calcium and phosphorus, appropriate to the species under study that allows a good calcium absorption at the intestinal balance to maintain homeostasis.[18]

The mean levels of magnesium concentration [Table 1] reduced when groups were evaluated with longer post-intervention. After the first 15 days of induction of critical defect, the average magnesium concentration was 3.1 mg/dL, which significantly reduced (P = 0.001) as compared that after 30 days of monitoring. However, the levels found during the experimental period were above the levels considered normal by Nichols[21] (1.6 mg/dL).

The relationship between bone mineral dose and the time of experimentation was not modified in any of the parameters, and remained 1.9-2.35% for calcium, 8.7-9.8% for phosphorus, and 0.17-0.21% for magnesium throughout the experimental period.

The ratio of bone mineral measurement with a time of trial does not change in any of the parameters, remaining 1.9-2.35% for calcium, 8.7-9.8% for phosphorus, and 0.17-0.21% for magnesium throughout the experimental period. Noteworthy is that, at 60-day follow-up, there was a reduction in the percentage of phosphorus and magnesium and an increase in the percentage of calcium.

Micrographs stained with hematoxylin-eosin of control animals [Figure 2] at 15 days postoperatively and treated [Figure 3] at 30-day postoperatively showed loose connective tissue with inflammatory infiltrate acute presence of fibroblasts and bone tissue with osteocytes, with no change in cellularity. It was also demonstrated that complete removal was promoted by loose connective tissue [Figures 2 and 3] that "hijacked" the implanted material, preventing direct contact of material with the bone interface and thereby hindering the stimulus that could be promoted by the action of the lectin to change cellularity. Therefore, the use of proteins that bind to carbohydrate residues has been proposed for the development and modulation of events such as communication, cellular differentiation, and proliferation.[22,23]

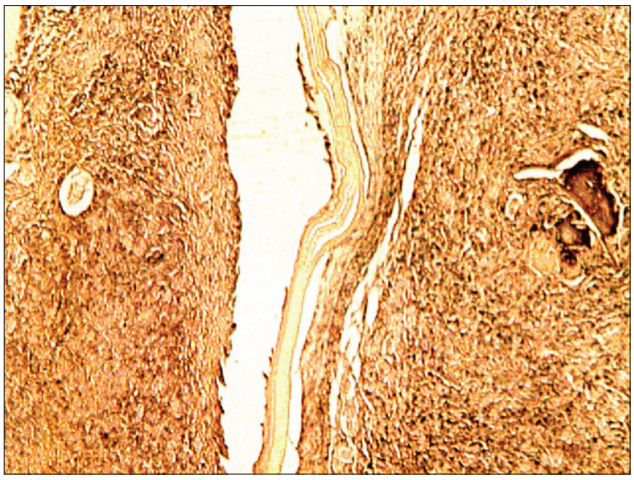

Figure 2: Micrograph of bone tissue of rats submitted to the critical defect in the parietal bone (H and E stain, ×100): Control animal group at 15 day postoperatively

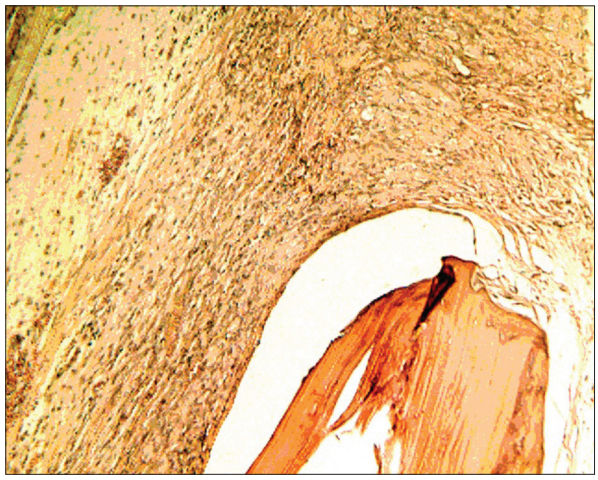

Figure 3: Micrograph of bone tissue of rats submitted to the critical defect in the parietal bone (H and E stain, ×100): Treated animal group at 30 day postoperatively

The principle of bone regeneration is that the process of bone repair occurs through competition between bone cells and connective tissue colonization and aimed at filling the critical defect, which represents one of the principles of tissue engineering.[24]

According to Ferreira *et al.*,[25] evaluating the ability of bone repair in critical calvarial defect in rats using a pool of bovine bone morphogenetic proteins linked to synthetic absorbable microgranular hydroxyapatite, histogicamente observed that in periods 1 to 3 months, the defects were filled with fibrous connective tissue and numerous foci of granulomatous foreign body around clusters of hydroxyapatite that inhibited bone formation.

According to Almeida *et al.*,[26] bringing non-osteogenic cells in the area of the surgical defect prevents the migration of cells with osteogenic potential, directly interfering in the process

of bone repair. Similar finding we observed in another study by Almeida *et al*.,[27] that evaluated the effect of *Plumbum metallicum* 30CH, a homeopathic medicine, on bone repair in jaws of rats without using a mechanical barrier and in the presence of muscle tissue in various histological sections.

The degree of damage to the loose connective tissue adjacent to the bone defect interferes with the experimental model of bone injury.[28] Although the loose connective tissue and animal species in the study area is very slim, the same degree of interference in the repair of bone tissue was extremely high, preventing the direct action of Cramoll-1,4 on bone tissue.

Conclusion

It is concluded that there were no significant changes in the biochemical serum and bone in relation to the effect of induced trauma. Despite the proven efficacy of the use of lectins in experiments on skin repair, with respect to the repair of bone tissue, Cramoll-1,4 (in irradiated gel formulation at a concentration of 300 μg) did not cause stimulation favoring the bone repair process, since there was direct interference of repair connective tissue that prevented direct contact of the implant with the bone interface.

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