

Effects of gamma irradiation on mechanical behavior and calcification of glutaraldehyde-fixed bovine pericardium

Efeitos da radiação gama no comportamento mecânico e na calcificação do pericárdio bovino fixado com glutaraldeído

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Abstract

Objective: To evaluate the effect of gamma irradiation on glutaraldehyde-fixed bovine pericardium.

Method: Glutaraldehyde-fixed bovine pericardium was exposed to gamma radiation (doses from 0 to 10000 Gy). Six samples from each of nine groups were evaluated by optic microscopy, and shrinking and mechanical tests and the denaturation temperature was determined. Additionally, they were subcutaneously implanted in rats and after four months they were explanted and Ca²⁺ levels measured by atomic absorption spectroscopy.

Results: The Ca²⁺ levels were (in µg/mg): control (0 Gy) - 194.45; 50 Gy - 154.64; 100 Gy - 169.37; 200 Gy - 163.64; 500 Gy - 199.89; 1000 Gy - 184.02; 2000 Gy - 198.95; 5000 Gy - 227.95; 10000 Gy - 362.62. Gamma irradiation caused a

significant effect on the biomechanical properties of the tissue.

Conclusion: Exposure to gamma irradiation did not reduce Ca²⁺ levels and caused a significant reduction in the tensile strength of glutaraldehyde-fixed bovine pericardium.

Descriptors: Glutaryl. Calcinosis. Gamma rays. Pericardium, radiation effects.

Resumo

Objetivo: Avaliar o comportamento mecânico e os efeitos no processo de calcificação pós-implante do pericárdio bovino tratado com glutaraldeído submetido a várias doses de radiação gama.

Método: Pericárdios bovinos fixados com glutaraldeído foram submetidos a radiação gama, nas doses de 0 a 10000

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Gy. Seis amostras de cada grupo foram avaliadas pela microscopia óptica, determinação da temperatura de desnaturação do colágeno e ensaio mecânico de tração e implantadas subcutaneamente em ratos. Após quatro meses do implante, as amostras foram explantadas e o conteúdo de Ca^{2+} determinado pela espectrometria de absorção atômica.

Resultados: Níveis de Ca^{2+} (em $\mu\text{g}/\text{mg}$): 0 Gy (controle) - 194,45; 50 Gy - 154,64; 100 Gy - 169,37; 200 Gy - 163,64; 500 Gy - 199,89; 1000 Gy - 184,02; 2000 Gy - 198,95; 5000 Gy - 227,95 e 10000 Gy - 362,62. Houve alteração significativa no comportamento mecânico do tecido irradiado, quando

comparado ao grupo controle, mesmo com o emprego de baixas doses de radiação.

Conclusão: O emprego da radiação gama no pericárdio bovino tratado com glutaraldeído não reduziu os níveis de Ca^{2+} em implantes subcutâneos em ratos por quatro meses e promoveu alteração significativa no comportamento mecânico do tecido, com redução na sua resistência, quando comparados ao grupo controle.

Descritores: Glutaral. Calcinose. Raios gama. Pericárdio, efeitos de radiação.

INTRODUCTION

Collagenous tissues treated with glutaraldehyde have been used in manufacturing heart bioprostheses however long-term post-implantation calcification constitutes a major cause of failure.

Glutaraldehyde treatment does not eliminate all metabolic activity of collagenous tissues. A preservation of 10 to 75% hydrolytic activity of the alkaline phosphatase enzyme has been demonstrated [1], suggesting its involvement in the process of calcification.

Calcification is an event with multiple causes involving factors related to the host and implant and to biomechanical aspects, starting with the formation of hydroxyapatite crystals in cellular membranes, which contain high phospholipid concentrations and much alkaline phosphatase enzyme activity [2].

The investigation of calcification, its control and how to delay its formation has been the focus of studies over the last two decades [3-7], many of which aimed at reducing the enzymatic activity of alkaline phosphatase either by its extraction or inhibition [8-15].

There are no reports in the literature on the effect of gamma radiation on calcification of glutaraldehyde-fixed bovine pericardium, however, changes in the spatial conformation of alkaline phosphatase induced by gamma radiation may, in theory, inhibit its enzymatic activity and interfere in the calcification process.

The aim of the current investigation is to evaluate the effects of different doses of gamma radiation on the mechanical behavior and post-implantation calcification process of glutaraldehyde-fixed bovine pericardium compared with a control group that was not exposed to radiation.

METHOD

Bovine pericardium was collected in a slaughterhouse after being examined by the Federal Inspectors of the

Ministry of Agriculture and Health Inspectors of the State Health Department immediately after the slaughtering of the animal. Pericardium was selected after an initial cleaning and removal of adipose tissue and transported in 0.9% NaCl and 1.0g MgSO_4 saline solution (pH 7.4) at 4°C. In the laboratory, the bovine pericardium was cleaned again and four samples with similar thicknesses and appearances were selected. These pericardia were placed in 15.0 x 12.0 cm plastic oval frames.

The pericardia were fixed in a 0.5% aqueous solution of glutaraldehyde buffered at pH 7.4 (phosphate buffer) and maintained in the dark for 10 days at 4°C with substitution of the solution on the 1st, 4th and 7th days. On finishing the fixation process, the pericardia were removed from the plastic frames, rinsed in a 0.9% NaCl saline solution under continuous stirring again exchanging the solution on several occasions and split into 9 groups to be submitted to gamma radiation: Group 1: 0 Gy (control); Group 2: 50 Gy; Group 3: 100 Gy; Group 4: 200 Gy; Group 5: 500 Gy; Group 6: 1000 Gy; Group 7: 2000 Gy; Group 8: 5000Gy and Group 9: 10000 Gy.

Determination of the radiosensitivity of alkaline phosphatase enzyme

Although alkaline phosphatase with hydrolytic activity can be extracted from fresh bovine pericardium, it can not be chemically detected after fixation for 24 hours in glutaraldehyde [16].

On considering this technical difficulty, we decided to evaluate the *in vitro* radiosensitivity of alkaline phosphatase in an aqueous solution of the pure enzyme with known activity and the remaining activity measured in respect to the different doses of gamma radiation. This solution was fractionated in nine aliquots, placed in glass containers and submitted to gamma radiation in a gamma chamber (source: Co^{60} , activity 22413.1×10^{10} Bq and dose rate 5010 Gy/h), in the following continuous doses: 0 Gy; 50 Gy; 100 Gy; 200 Gy; 500 Gy; 1000 Gy; 2000 Gy; 5000 Gy and 10000 Gy.

The resulting activity of the alkaline phosphate in each aliquot was determined by the formation of p-nitrophenol with readings of the absorbance in a spectrophotometer using a wavelength of 450 nm.

Determination of shrinking temperature

The temperature of abrupt shrinking, that is, the temperature required to release H-bonds from the triple helices and randomly modify the structure of collagen, was employed to determine the degree of stability of the tissue after fixation.

Five samples from each group, cut in 3.0 x 1.0 cm strips and immersed in a water-bath with 0.9% NaCl saline solution were placed in a way so that one end was fixed at the bottom of the apparatus and the other submitted to traction of 0.5 g. The temperature of the water-bath was progressively increased by 4°C/min until abrupt shrinking of the sample occurred.

Traction test

Five samples of each group were submitted to traction in an INSTRON model 4400R apparatus to determine stretching and tension.

The samples were cut into 3.0 x 0.4 cm strips, placed in the clamps of the apparatus and maintained immersed in 0.9% NaCl saline solution at a constant temperature of 37°C. An initial force of 0.5g was applied and the test was performed at a steady velocity of 10 mm/min until the sample ruptured.

Force vs. displacement curves were drawn. To calculate traction tension the initial area (width x thickness) of the samples was considered and the stretching was determined by displacement of the apparatus clamps. To determine the thickness, samples were placed between two glass slides and measured using a digital pachymeter.

Stretching was measured up to a point equivalent to the maximum tension sustained by the tissue and the mechanical resistance was defined as the value of the highest supported tension.

The curve selected for analysis as being representative of each group was the one that was closest of the mean value of the maximum tensions.

Control of sterility

The sterility of the samples was guaranteed by means of culture tests with samples being left in an incubator at 25°C for 14 days with Thioglycolate and Sabouraud as culture mediums.

Quantitative measurement of Ca²⁺

Five samples from each group were dehydrated in an incubator at 50°C and mineralized in a Mufla stove at 800°C.

Mineralized samples were dissolved in 2.5M HNO₃ and sent for quantitative measurement of the Ca²⁺ by atomic absorption spectroscopy in a Perkin Elmer spectrometer (Analyst 100 model) using a wavelength of 422.7nm. The calibration curve was obtained using a standard 1.000mg/L Perkin Elmer solution with the addition of 1% lanthanum chloride. The total quantity of Ca²⁺ was expressed as µg per mg of dry tissue.

Implantation in rats

Twenty-seven female Wistar rats, with a mean age of 30 days, were utilized in this experiment. The animals were obtained from the animal house of the Institute of Energy and Nuclear Research and the research was approved by the Ethics Research Commission of the institution. The established norms in the Guide for the Care and Use of Laboratory Animals and the ethical principles on animal experimentation of the Brazilian College of Animal Experimentation were respected.

The rats were anesthetized, trichotomized and samples of the tissue, measuring 2.0 x 1.5 cm were implanted at two dissected subcutaneous points in the dorsal region, giving a total of six samples for each group. After four months, the samples were explanted, dissected to remove the host animal tissue and washed in a 0.9% NaCl solution. Five samples of each group were sent for Ca²⁺ measurement and the remainder was assessed by optical microscopy after von Kossa staining.

Statistical analysis

The data are reported as means ± standard deviation. The differences between the means of groups were tested using the Student t-test. The results were considered significant when p<0.05.

RESULTS

Radiosensitivity of the alkaline phosphatase enzyme

The inhibition of the enzymatic activity increased as the doses of gamma radiation increased with a reduction of more than 98% of its initial activity with doses equal to or greater than 5000 Gy (Figure 1).

Optical microscopy

In pre-implantation irradiated samples, preservation of a homogenous collagen structure was observed. In explanted samples, there was intense calcification in all groups (Figure 2).

Temperature of shrinking

In all samples, the temperature was greater than 82°C (value considered adequate for fixed bovine pericardium) independent of the dose of radiation employed.

Mechanical behavior

Significant changes occurred in the mechanical behavior of the irradiated tissue which demonstrated greater deformation and reduced resistance when compared to the Control Group. The tension-deformation curves representative of each group are shown in Figure 3.

Mineral analysis

A reduction in calcium levels was not observed in the irradiated samples and there was a significant increase of the Ca²⁺ content of samples submitted to doses of 10000 Gy when compared to the Control Group (p < 0.05). The quantity of Ca²⁺ obtained in samples of the different groups, pre- and post-implantation, are illustrated in Table 1.

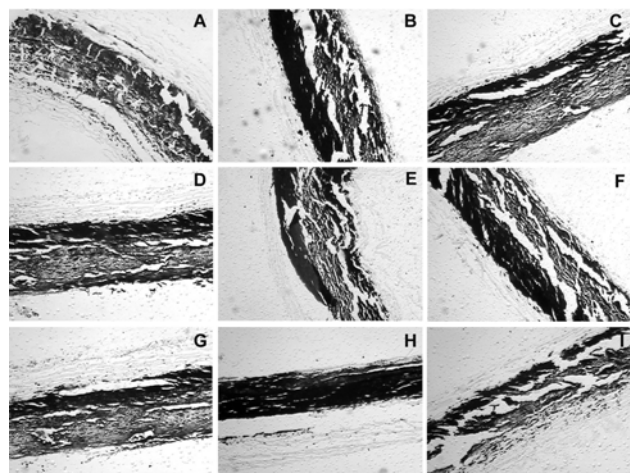


Fig. 1 – Micrographs of explanted bovine pericardium samples. A. 0 Gy; B. 50 Gy; C. 100 Gy; D. 200 Gy; E. 500 Gy; F. 1000 Gy; G. 2000 Gy; H. 5000 Gy; I. 10000 Gy - Von Kossa stain, magnification 80x

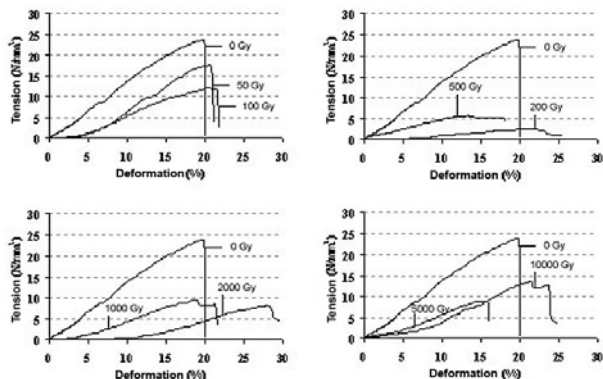


Fig. 2 – Tension x deformation curves correlated to the dose of gamma irradiation used (0 Gy = Control Group)

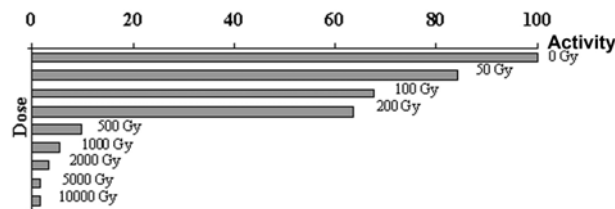


Fig. 3 – Hydrolytic activity of alkaline phosphatase enzyme in aqueous solution correlated to the dose of gamma irradiation used (n=5)

Table 1. Measurement of Ca²⁺ in pre- and post-implanted samples correlated to the dose of gamma irradiation used

Dose	Pre-implantation		Post-implantation	
	Ca ²⁺ (µg/mg)	σ (µg/mg)	Ca ²⁺ (µg/mg)	σ (µg/mg)
0 Gy	0.66	0.02	194.45	19.50
50 Gy	0.77	0.04	154.64	34.36
100 Gy	0.64	0.04	169.37	30.77
200 Gy	0.75	0.02	163.64	27.55
500 Gy	0.73	0.02	199.89	81.97
1000 Gy	0.95	0.02	184.02	44.10
2000 Gy	0.66	0.03	198.95	49.95
5000 Gy	0.69	0.04	227.95	54.24
10000 Gy	0.78	0.02	362.62	20.27

DISCUSSION

There have been few publications on the effects of gamma radiation on collagen with the majority restricted to fresh collagen.

Cassel [17], using doses of 5 to 220 x 10⁴ Jkg⁻¹ on bovine skin and kangaroo tail tendon, observed decreases in the shrinking temperature and increases in the solubility and concluded that gamma radiation causes a breakdown of peptic chains.

Bowes and Moss [18] studied the effects of gamma irradiation on purified collagen obtained from bull's tails. In dry irradiated collagen, they observed an accentuated change in the resistance to traction, even at low doses, reduction in shrinking temperature and increase in solubility. In irradiated collagen in liquid, they observed shrinking temperatures greater than 100°C and a slight alteration in the solubility.

The effects of gamma irradiation on the structure of collagen treated with glutaraldehyde were described by Grant et al. [19], who observed, at electronic microscopy, a significant protection against the destructive effects of

gamma irradiation with doses of up to 25×10^4 JKg¹.

Hafeez et al. [20] studied the mechanical proprieties of fresh bovine pericardium submitted to 25 kGy of gamma irradiation and observed changes in the mechanical proprieties of tissue with a significant reduction in the resistance.

In our investigation, the alkaline phosphatase in aqueous solution was highly radiosensitive and, although the enzymatic behavior in respect to gamma radiation has not been previously described, these results were expected as inhibition of the activity of other enzymes using gamma irradiation has already been published [21, 22].

Structural integrity of the fixed collagen in pre-implanted samples was not modified by gamma radiation however there was a significant change in the mechanical behavior of irradiated tissue when compared with the Control Group, even with low doses of radiation. The deformities were greater and the mechanical resistance was less than those observed in the Control Group and not proportional to the doses used.

In spite of accentuated radiosensitivity of the alkaline phosphatase enzyme, with a reduction in its initial activity of more than 98% in doses of 5000 Gy, there was no reduction in the calcification of irradiated tissue and at doses of 10000Gy, there was a significant increase in Ca²⁺ levels ($362.61 \pm 20.27 \mu\text{g}/\text{mg}$ vs. $194.45 \pm 19.50 \mu\text{g}/\text{mg}$ in the Control Group).

Although the data obtained in an aqueous solution of pure enzyme can not be automatically transferred to the fixed tissue, it is possible to deduce that gamma irradiation does not have anticalcifying properties in bovine pericardium treated with glutaraldehyde.

CONCLUSION

The use of gamma radiation in bovine pericardium treated with glutaraldehyde resulted in an increase in Ca²⁺ levels in subcutaneous implantations in rats over four months and promoted a significant alteration in the mechanical behavior of the tissue, with a reduction in the resistance when compared to the Control Group.

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