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Original article

Calcitonin as an anticalcification treatment for implantable biological tissues

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ABSTRACT

Background and aim of the study: Calcification remains the major role of failure of implantable biomedical material and in particular of bioprosthetic valves. Various treatments have been proposed to mitigate calcification of glutaraldehyde-fixed bioprosthetic valves but none have succeeded in inhibiting or mitigating efficiently the calcification process of the implantable biological tissues. Since the discovery of calcitonin (CT) and its therapeutic role in treating hypercalcemic patients, CT has never been tried as an anticalcification treatment for biomaterials. It is postulated, that tissue calcification may be efficiently minimized by forming adducts with aldehyde groups thus eliminating the places of the biological tissues onto the calcium cations could be deposited.

Material and methods: Fresh porcine aortic leaflets were cut radially in three parts. Three groups of tissue were created. Group I (glutaraldehyde only), Group II (glutaraldehyde with 1% CT) and Group III (glutaraldehyde with 10% CT). All tissues were then implanted subdermally in three sets of 8 (Group I) and 9 (Group II and Group III) male Wistar rats of 12 days old. 21 days later the rats were euthanized by inhalation of CO_2. The tissues were retrieved and after rinsing with distilled water 3 times, were lyophilized at −40 °C at high vacuum pressure of approximately 100 mmHg for 16 h. The calcium content was then measured with flat atomic absorption technique.

Results: The preimplantation values of Ca concentration as expressed in mg Ca/g of tissue were 1.79 ± 0.14 in Group I, 4.78 ± 0.0079 in Group II and 2.88 ± 0.17 in Group III (p = ns). 21 days later the values of Ca concentration were 126.95 ± 12.97 for Group I, 24.69 ± 2.71 for Group II ($p < 0.05$) and 27.16 ± 2.95 for Group III ($p < 0.05$). There was not significance difference between Groups II and III, even if Group II showed a less accumulation of Ca concentration (×5.16) than Group III (×9.43).

Conclusion: An anticalcification treatment based on calcitonin as an additive to buffered glutaraldehyde, mitigates the calcification process of the implantable biological tissues, as compared to glutaraldehyde treatment only.

Introduction

Biological tissues are used in medicine over the last four decades, for constructing bioprosthetic heart valves, closing cardiac defects or used as arterial conduits. The tissues can either be of porcine, bovine or equine origin [1,2], even if other animal sources have been proposed as well [3–5]. Being xenografts, the tissues need to become immunologically inert, sterilized and preserved and this is succeeded mainly by glutaraldehyde fixation [6]. This treatment though with glutaraldehyde make the tissues susceptible to calcification which remains the major role of failure of implantable biomedical material and in particular of bioprosthetic valves [7]. A strong relationship exists between inflammatory infiltration from macrophages and calcification in glutaraldehyde-fixed bioprosthetic valves [8].

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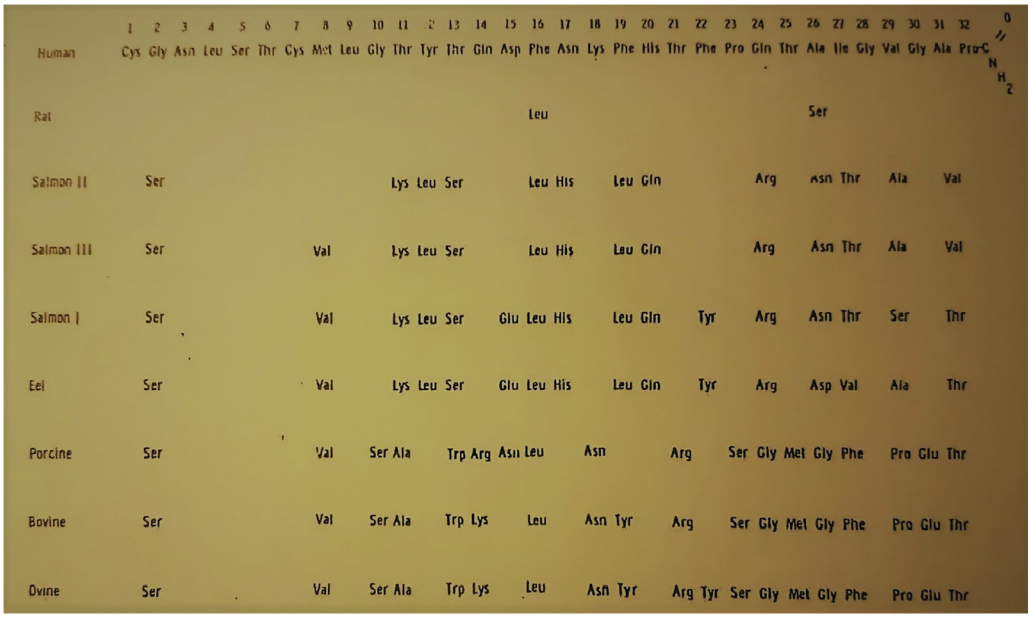

Fig. 1. Amino acid sequel of human Calcitonin single helix and the differences between Various species. (From EA Agathos, PhD Thesis [11]).

Calcitonin is a 32-amino acid linear polypeptide hormone (Fig. 1), with a molecular weight of 3454.93 Da, that is produced in humans primarily by the parafollicular cells (also known as C-cells) of the thyroid, and in many other animals in the ultimopharyngeal body [9]. It acts to reduce blood calcium (Ca^{2+}), opposing the effects of parathyroid hormone (PTH) [10,11].

Various treatments have been proposed to mitigate calcification of glutaraldehyde-fixed bioprosthetic valves but none have succeeded in inhibiting efficiently the calcification process of the implantable biological tissues. At this point we wanted to investigate the role of Calcitonin, when used as an additive to the glutaraldehyde fixation and preservation of the biological tissues, serving as a possible anticalcification treatment.

Materials and method

The method comprises of placing the biological tissue for fixation and preservation in a buffered glutaraldehyde (Glut) solution 0.5% at pH 7.4, adding salmon synthetic calcitonin (CT), Miacalcic® of Novartis Hellas A.B.E., at two different concentrations. One with 1 unit/100 ml (1%) and the other with 10 units/100 ml (10%). The first fixation process lasted 1 h, at a pressure between 2 and 3 mmHg and subsequently the biological tissues were embedded in an accordingly new solution for 1 week.

Porcine aortic leaflets were selected as fresh tissue from a local slaughter house and were cut radially in three parts. Following harvesting, the biological tissue is rinsed and maintained in cold Hepa saline solution (temperature between 4–12 °C) between 2 and 6 h. The Hepa saline solution can be made by the following formula: 20 l of distilled water (Millipore-Direct-Q^{TM} (18.2 MΩ cm) with 180.01 g NaCl, 19.73 g $NaHPO_4$, 121.72 g Na_2HPO_4, 20 ml HCl 1 M (pH 7.4).

Three groups of tissue were created. Group I (Glut only), Group II (Glut with 1% CT) and Group III (Glut with 10% CT). All tissues were then implanted subdermally in three sets of 8 (Group I), 9 (Group II) and 9 (Group III) male Wistar rats of 12 days old (Center for Experimental Surgery, Biomedical Research Foundation of the Academy of Athens). The rats were selected along with their mother and had a free alimentation regime. All tissues were rinsed three times in normal saline solution for 10 min each time before implantation. Each rat received four fragments of tissue at the dorsum, through four separate incisions (two at each side) each of 1 cm long, with a technique we have previously described elsewhere [8].

21 days later the rats were euthanized by inhalation of CO_2. All procedures were approved by the Animal Care Committee of the Academy of Athens and performed according to the Guide for the Care and Use of Laboratory Animals prepared by the Institutes of Laboratory Animal Resources, National Research Council and published by the National Academy Press, revised 1996 (NIH publication No. 85-23). The tissues were retrieved and after rinsing with distilled water 3 times, were lyophilized at −40 °C at high vacuum pressure of approximately 100 mmHg for 16 h. The calcium content was then measured with flat atomic absorption technique.

For statistical analysis, the commercially available software package ANOVA Origin 8.0 for Windows (OriginLab Corporation, Northampton, Massachusetts, USA) was used. *P* values of 0.05 or less were defined as a statistically significant difference.

Results

The pre-implantation values for mg Ca/mg tissue of the various groups are listed in Table 1. Group I (control group) represents glutaraldehyde fixed tissues without anticalcification treatment, while Group II represents samples treated with buffered 1% CT solution and Group III represents samples treated with 10% CT solution.

The post implantation weight of the samples along with the values for mg Ca/mg tissue of the various groups are listed in Table 2, while Table 3 shows the cumulative results of Ca concentration in the various group and the statistical differences. Fig. 2 gives a diagrammatic comparison of Ca Content in the three groups showing the significant difference of Ca concentration in Group I versus Group II and Group III.

There was not significance difference between Groups II and III, even if Group II showed a less Ca concentration accumulation (×5.16) than Group III (×9.43) in the explanted tissues. All numeric data were expressed as mean ± standard deviation (SD).

Table 1
Pre implantation Ca content.

	Weight (g)	mg Ca/g tissue
Group I		
1	0.0045	1.79
2	0.0033	1.87
3	0.0039	1.60
4	0.0047	1.98
5	0.0031	1.71
Mean	0.0039	1.79
STDEV(±)	0.0007	0.14
Group II		
1	0.0105	4.73
2	0.0110	4.83
3	0.0115	4.88
4	0.0102	4.78
5	0.0118	4.68
Mean	0.0110	4.78
STDEV(±)	0.0007	0.079
Group III		
1	0.0120	3.12
2	0.0112	2.64
3	0.0117	2.90
4	0.0128	2.88
5	0.0123	2.86
Mean	0.0120	2.88
STDEV(±)	0.0006	0.17

No statistical difference between the various groups. STDEV: standard deviation.

Table 2
Post implantation Ca content.

	Weight (g)	mg Ca/g tissue
Group I		
1	0.0075	147.40
2	0.0094	120.60
3	0.0112	114.00
4	0.0117	127.32
5	0.0070	144.68
6	0.0071	119.70
7	0.0073	130.62
8	0.0079	111.33
Mean	0.0086	126.95
STDEV(±)	0.0009	12.97
Group II		
1	0.0380	4.78
2	0.0280	25.53
3	0.0830	25.71
4	0.0234	20.84
		27.78
5	0.0360	22.08
6	0.0480	28.23
7	0.0360	26.11
8	0.0410	21.34
9	0.0790	24.56
Mean	0.0458	24.69
STDEV(±)	0.0212	2.71
Group III		
1	0.0480	29.58
2	0.0400	30.25
3	0.0470	29.79
4	0.0600	26.83
5	0.0500	21.70
6	0.0360	28.47
7	0.0400	25.00
8	0.0430	28.60
9	0.0440	24.20
Mean	0.0453	27.16
STDEV(±)	0.0071	2.95

STDEV: standard deviation.

Table 3
Cumulative results of post-implantation Ca concentration in the various group and statistical difference.

Group I (Glut only) 8 rats	Group II (1% CT) 9 rats	Group III (10% CT) 9 rats
126.95 ± 12.97	24.69 ± 2.71 $p < 0.05^{*}$	27.16 ± 2.95 $p < 0.05^{**}$
		$p = \text{ns}^{***}$

Group I: Glutaraldehyde only; Group II: 1% Calcitonin; Group III: 10% Calcitonin.
* The statistical difference between Group II and Group I.
** The statistical difference between Group III and Group I.
*** The statistical difference between Group II and Group III.

Discussion

Glutaraldehyde fixation effects the cross-linking of the collagen within the tissue. Such cross-linking tends to make the tissue more durable. The cross-linked protein exhibits increased resistance to proteolytic cleavage, which involves unfolding of the protein substrate in order to facilitate enzymatic hydrolysis. Cross-linking of the protein makes the tissue resistant to such unfolding, thus preventing deterioration due to the enzymatic activity of blood.

Residual aldehydes groups within glutaraldehyde-fixed tissues serve as the points where calcification occurs. Different agents that are capable of blocking residual aldehydes – such as sodium bisulfite [12], L-glutamic acid [13], glycine [14], L-lysine [15], diamine [16] and L-arginine [17] have been used to mitigate calcification in glutaraldehyde-fixed tissue, demonstrating significant reductions in the in vivo deposition of both calcium and phosphorus.

The discovery of calcitonin (CT) was first made by Copp et al. as a result of perfusing isolated thyroid-parathyroid gland preparations in the anesthetized dog [18]. Pearse showed that the origin of CT was the C-cells of the thyroid gland [19]. Potts et al. determined the amino acid sequence of human and salmon CT, which led to the synthesis and commercial use of the more potent salmon CT [20].

Since the discovery of CT, several potential roles of action have been suggested. CT has been shown to decrease the magnitude of hypercalcemia during calcium loading [21] and increase the renal

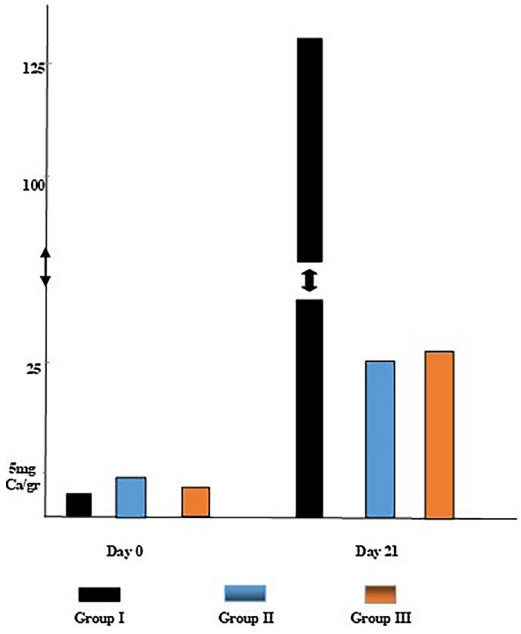

Fig. 2. Diagrammatic comparison of Ca content in the three groups.

production of 1,25D [22]. During pregnancy and lactation, both 1,25D and CT levels are increased [23]. The effectiveness of CT in the treatment of hypercalcemia is attributed to reducing osteoclast activity. Besides decreasing osteoclast activity, CT has been suggested to facilitate deposition of calcium and phosphorus in bone especially in the post-prandial state [24].

Gender and age differences in CT have been shown, with women having lower values than men and with decreasing CT values with age. CT has a definite strong relationship with the transient hypocalcemia observed soon following total thyroidectomies in human patients [11]. CT screening has been shown to be a useful tool for the diagnosis of medullary thyroid carcinoma [25]. However, despite several attractive and plausible hypotheses, CT has not been shown to have an important physiologic role in humans more than 50 years after its discovery.

Our findings reveal a new property of calcitonin, that of an anticalcification agent during the fixation and preservation of implantable biological tissues. Even if the mechanism is still unclear, it is speculated that CT may offers a local protection by forming a chemical bond with the free aldehyde groups of glutaraldehyde. Smaller concentration of CT showed less Ca accumulation, even if this difference was not statistical significant. Both concentrations of 1 and 10 units per 100 ml were chosen arbitrarily, based on the availability of the commercialized Calcitonin, which comes as 100 units per 1 ml. Fraction of the unit such as 0.1% or less is something that the scientific community can investigate in the future.

Even if we did not measured the circulating levels of native Calcitonin, PTH and thyroid hormones of the experimental animals, we speculate that these were not affected, as the animals were growing and behaving normally for their age. We believe that the fixed into the preserved tissues small amount of Calcitonin is not released into the systemic circulation due to the aforementioned chemical bond. Histological studies of the specimens were not included in this study, as we were focused on measuring Ca content only, and we cannot comment on how macrophages or other inflammation elements react with Calcitonin treated tissues.

Studying artificial valves in a clinical setting is difficult, this is why tissue samples have been subdermally implanted into rats. This method has some drawbacks as there is no direct contact between the experimental tissues and circulating blood; nevertheless, it has been deemed acceptable for studying xenograft calcification [26]. More study of this observation will be needed to clarify efficiently the exact mechanism of the anticalcification effect and the long term clinical effects if any in the living animals. Longer implantation time in vivo, along with mechanical stress testing of the tissue and histological examination may be necessary to demonstrate the effectiveness and durability of this anticalcification treatment over the existing commercial ones.

Conflict of interest

None declared.

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References

[1] Jameison WRE. Modern cardiac valve devices— bioprostheses and mechanical prostheses: state of the art. J Cardiac Surgery 1993;8:89–98.
[2] Mueller Xavier M, von Segesser Ludwig K. A new equine pericardial stentless valve. J Thorac Cardiovasc Surg 2003;125:1405–11.
[3] Weinhold C, Weingartner J, Adt M, Peters D, Reichart B, Kreuzer E, et al. Kangaroo xenobioprostheses: an advantage in heart valve replacement. Z Kardiol 1986;75(Suppl 2):251–3.
[4] Agathos EA, Shen M, Styrc W, Giannakopoulou S, Lachanas E, Tomos P. Hydrodynamic performance of a prototype bioprosthetic valve derived from the pulmonary valve of Phoca Groenlandica. ASAIO J 2012;58(5):535–9.
[5] Agathos EA, Tomos P, Lachanas E, Gakiopoulou H, Pantopoulou A, Perrea D. Experimental replacement of pig trachea with novel bioprosthesis from Harp Seal. Asian Cardiovasc. Thoracic Ann. 2010;18(6):557–62.
[6] Carpentier A, Lemaigre G, Robert L, et al. Biological factors affecting long-term results of valvular heterografts. J Thorac Cardiovsc Surg 1969;58:467–83.
[7] Levy RJ, Schoen FJ, Levy JT, et al. Biological determinants of dystrophic calcification and osteocalcin deposition in glutaraldehyde-preserved porcine aortic valve leaflets implanted subcutaneously in rats. Am J Pathol 1983;113:143–55.
[8] Agathos EA, Shen M, Katsiboulas M, Koutsoukos P, Gloustianou G. In vivo calcification of glutaraldehyde fixed cardiac valve and pericardium of Phoca Groenlandica. ASAIO 2011;57(4):328–32.
[9] Niall HD, Keutmann HT, Copp DH, et al. Amino acid sequence of salmon ultimobranchial calcitonin. Proc Natl Acad Sci USA 1969;64:771–8.
[10] Kanis JA, Heynen G, Cundy T, et al. An estimate of the endogenous secretion rate of calcitonin in man. Clin Sci 1982;63:145–52.
[11] Agathos EA. The role of Calcitonin and Parathormone during the transient hypocalcemia following total thyroidectomy, PhD Thesis. University of Athens Medical School; 1988. http://dx.doi.org/10.12681/eadd/9351.
[12] Zhou J, Jiang H, Wang D, Hu S. A novel anti-calcification strategy of bovine pericardium using sodium bisulfite modification. J Heart Valve Dis 2009;18:180–6.
[13] Grimm M, Eybl E, Grabenwoger M, et al. Biocompatibility of aldehyde-fixed bovine pericardium. An in vitro and in vivo approach toward improvement of bioprosthetic heart valves. J Thorac Cardiovasc Surg 1991;102:195–201.
[14] Chanda J. Post-treatment with amino compounds effective in prevention of calcification of glutaraldehyde treated pericardium. Artif Organs 1994;18:408–10.
[15] Weissenstein C, Human P, Bezuidenhout D, Zilla P. Glutaraldehyde detoxification in addition to enhanced amine cross-linking dramatically reduces bioprosthetic tissue calcification in the rat model. J Heart Valve Dis 2000;9:230–40.
[16] Zilla P, Bezuidenhout D, Weissenstein C, van der Walt A, Human P. Diamine extension of glutaraldehyde crosslinks mitigates bioprosthetic aortic wall calcification in the sheep model. J Biomed Mater Res 2001;56:56–64.
[17] Jee KS, Kim YS, Park KD, et al. A novel chemical modification of bioprosthetic tissues using L-arginine. Biomaterials 2003;24:3409–16.
[18] Copp DH, Cheney B. Calcitonin—a hormone from the parathyroid which lowers the calcium-level of the blood. Nature 1962;193:381–2.
[19] Pearse AG. The cytochemistry of the thyroid C cells and their relationship to calcitonin. Proc R Soc London B Bio Sci 1966;164:478–87.
[20] Potts Jr JT. Chemistry of the calcitonin. Bone Miner 1992;16:169–73.
[21] Rodriguez M, Felsenfeld AJ, Torres A, et al. Calcitonin, an important factor in the calcemic response to parathyroid hormone in the rat. Kidney Int 1991;40:219–25.
[22] Jaeger P, Jones W, Clemens TL, et al. Evidence that calcitonin stimulates 1,25-dihydroxyvitamin D production and intestinal absorption of calcium in vivo. J Clin Invest 1986;78:456–61.
[23] Kumar R, Cohen WR, Silva P, et al. Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation. J Clin Invest 1979;63:342–4.
[24] Heersche JNM. Calcitonin effects on osteoclastic resorption: the 'escape phenomenon' revisited. Bone Miner 1992;16:174–7.
[25] Costante G, Meringolo D, Durante C, et al. Predictive value of serum calcitonin levels for preoperative diagnosis of medullary thyroid carcinoma in a cohort of 5817 consecutive patients with thyroid nodules. J Clin Endocrinol Metab 2007;92:450–5.
[26] Dewanjee MK, Solis E, Mackey ST, et al. Quantification of regional platelet and calcium deposition on pericardial tissue valve prostheses in calves and effect of hydroxyethylene diphosphate. J Thorac Cardiovasc Surg 1986;92:337–48.