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Evaluation of bovine pericardial regenerative membrane in the presence and absence S9 activation using Ames test

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Abstract:
Bovine pericardial membranes are widely used in guided tissue regeneration (GTR). However, their genetic safety has not been fully established. Therefore, it is of interest to evaluate the genotoxicity of bovine pericardial as a GTR material in the presence and absence of S9 activation in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, at concentrations of 0.3125–5 mg/plate using the Ames test. We found no biologically relevant increase in revertant colonies. Thus, bovine pericardial is non-mutagenic and supports its preclinical genetic safety for periodontal regeneration applications. Further *in vivo* studies are warranted to confirm its long-term safety.

Keywords: Genotoxicity, mutation assay, bovine pericardial, guided tissue regeneration (GTR)

Background:
Biomaterials are classified as inert, bioactive, or bioresorbable according to their tissue response **[1]**. They should exhibit biocompatibility and appropriate load-bearing capacity, hardness and flexibility for clinical applications, including orthopedic and cardiovascular therapies **[1].** Materials used for guided tissue regeneration (GTR) should be safe, effective, biocompatible and cost-efficient. However, each material presents specific advantages and limitations **[2].** Barrier membranes play a key role in GTR for periodontal repair. They promote regeneration of the periodontal ligament, cementum and alveolar bone by preventing soft tissue proliferation into the defect area **[3].** The interaction between bovine pericardium membranes and human periodontal ligament fibroblasts has been investigated to evaluate their regenerative potential **[4].** Genotoxicity testing is essential for verifying the biological safety of GTR materials. It identifies DNA damage, gene mutations and chromosomal abnormalities in accordance with ISO standards for medical devices **[5].** *Salmonella typhimurium* and *Escherichia coli* are widely used for detecting gene mutations because of their simplicity and sensitivity **[6].** However, positive bacterial findings do not necessarily predict comparable effects in eukaryotic cells or mammals **[6].** The Ames test, developed by Dr. Bruce Ames in 1971, employs genetically modified *Salmonella typhimurium* strains to assess mutagenicity **[7].** The assay shows approximately 83% concordance between mutagenicity and carcinogenicity, making it a valuable screening tool for genotoxicity **[7].** We reported no mutagenic effects of bovine pericardium in the Ames test with S9 metabolic activation **[8].** Therefore, it is of interest to report the synthesis, characterization and genotoxicity evaluation of a bovine pericardium-derived GTR membrane using the Ames test with and without S9 activation, providing preclinical evidence of its genetic safety and potential periodontal application.

Materials and Methods:
This study evaluated the genotoxic potential of bovine pericardium in periodontal applications using a using the Ames test in the presence and absence of S9 activation at the Ames Test Laboratory, School of Dental Sciences, USM. Bovine pericardial was stored aseptically and positive controls were used for accuracy. *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 were employed to detect mutations**.** The study used Vogel-Bonner Salts, glucose, histidine/biotin solutions, top agar and S9 homogenate with liver enzymes like cytochrome P-450 to assess mutagenicity **[9].**

Experimental material preparation:
Bovine pericardium (BP) was incubated in sterile water and tested for mutagenicity using the standard plate incorporation assay.

Positive controls preparation:
The positive controls were dissolved in distilled water and then stored at -80°C.

Methodology:
The Ames test was conducted using the pre-incubation method with bacterial strains TA98, TA100, TA1535 and TA1537, in the presence and absence of S9 activation. Triplicates for negative controls and duplicates for test and positive controls were evaluated.

Procedures:
The Ames test detects genetic mutations by exposing tester strains to the test substance in a pre-incubation assay for 20 minutes, followed by plating on glucose minimal agar **[10, 8]**. Pure water served as negative control, while chemical agents were used as positive controls.

Selection of dosages:
The study tested doses from 0.3125 to 5 mg/plate, showing no growth inhibition or increased revertant colonies in TA 100, TA 1535, TA 98 and TA 1537 strains, in the presence and absence of S9 activation.

Counting of bacterial colonies:
Bacterial colonies were counted manually or with a device and the mean of three enumerations was used for final calculation.

Table 1: Comparative bacterial counts in test and control in presence and absence of S9 activation

CO mg/plate	Without S9 Mean number of revertants/plate				RM	With S9 Mean number of revertants/ plate				RM
	TA 98	TA 1537	TA 100	TA 1535		TA 98	TA 1537	TA 100	TA 1535	
0.3125	186	247	246	382	No	300	227	274	305	No
0.625	267	225	269	215	No	223	314	294	333	No
1.25	214	324	299	325	No	248	253	277	351	No
2.5	144	224	328	246	No	211	286	159	486	No
5	274	330	282	332	No	294	337	287	308	No
NC	286	358	352	287	No	339	456	293	445	No
PC	587	860	680	1123	Yes	920	1031	607	898	Yes

Table 2: Comparative one-way ANOVA and post-hoc dunnett analysis

Bacterial Strain	Metabolic Activation	NC Mean (Baseline)	Test Groups Range (0.3125–5.0 mg/plate)	PC Mean (Positive Ctrl)	ANOVA Overall p-value	Post-Hoc Dunnett (p vs. NC)
TA 98	0	286	144 – 274	587	p = 0.392	All doses: ns (p > 0.05)
TA 98	0	339	211 – 300	920	p = 0.471	All doses: ns (p > 0.05)
TA 1537	0	358	224 – 330	860	p = 0.284	All doses: ns (p > 0.05)
TA 1537	0	456	227 – 337	1031	p = 0.178	All doses: ns (p > 0.05)*
TA 100	0	352	246 – 328	680	p = 0.667	All doses: ns (p > 0.05)
TA 100	0	293	159 – 294	607	p = 0.362	All doses: ns (p > 0.05)*
TA 1535	0	287	215 – 382	1123	p = 0.444	All doses: ns (p > 0.05)
TA 1535	0	445	305 – 486	898	p = 0.523	All doses: ns (p > 0.05)

Results and Discussion:
The study evaluated the genotoxic potential of bovine pericardium for guided tissue regeneration (GTR) using the bacterial reverse mutation (Ames) assay in the presence and absence of S9 activation, confirming that revertant colony counts did not exceed the threshold for mutagenic response relative to the negative control **(Table 1 and Table 2)**. NC**-** Negative Control, PC**-** Positive Controls, RM**-** Reverse Mutation, **CO-** Concentrations. The test substance, at all tested concentrations, did not induce a biologically relevant increase in revertant colonies in any strain, as the counts remained less than two-fold relative to the negative control, with and without S9 activation. Note: ns = not statistically significant ($p > 0.05$) versus the negative control. All positive control groups (PC) showed a highly significant increase in revertant counts ($p < 0.001$), validating assay sensitivity. Isolated statistical fluctuations below the baseline occurred due to minor random variance or mild, non-hazardous cytotoxicity; no concentration met the criteria for a mutagenic increase (*i.e.,* a sustained >= 2-fold elevation). Based on the One-Way ANOVA and post-hoc statistical evaluation, the test substance did not induce a biologically relevant or statistically significant increase in revertant colonies in any tester strain, presence and absence of S9 metabolic activation. Therefore, under the conditions of this assay, the test substance is concluded to be non-mutagenic. Initial evaluations of cytotoxicity and mutagenicity are essential during the development of biomaterials intended for implants and prostheses. In the present study, the bacterial reverse mutation (Ames) assay was employed to evaluate the genotoxic potential of bovine pericardial (BP) intended for guided tissue regeneration (GTR) in the presence and absence of S9 metabolic activation. The results showed that BP did not induce a biologically relevant or statistically significant increase in revertant colonies in any *Salmonella typhimurium* tester strain at any tested concentration. Revertant counts remained below the established threshold of a two-fold increase relative to the negative control under both S9-activated and non-activated conditions, while all positive controls produced highly significant increases ($p < 0.001$), confirming assay sensitivity and validity. Minor reductions in revertant counts observed in isolated groups were not statistically significant ($p > 0.05$) and most likely reflected normal biological variation or mild non-hazardous cytotoxicity rather than mutagenic activity. Collectively, these findings indicate that BP and its potential metabolites exhibit no detectable mutagenic activity under the experimental conditions employed, supporting its biological safety for potential medical applications **[11].** The Ames test is a widely accepted screening method for detecting mutagenic compounds and their metabolites and is recommended by OECD Test Guideline 471 **[9].** The reliability of the present findings is supported by adherence to internationally accepted testing protocols, including appropriate bacterial culture preparation, concentration selection, incubation procedures and metabolic activation **[6-8]**. Moreover, the use of four genetically distinct *Salmonella typhimurium* tester strains enabled the detection of both frameshift mutations (TA98 and TA1537) and base-pair substitutions (TA100 and TA1535), thereby providing a comprehensive assessment of mutagenic potential **[19, 20]**. The expected responses obtained with sodium azide, acridine orange, 4-nitro-*o*-phenylenediamine and 2-aminoanthracene

confirmed the physiological integrity and sensitivity of the tester strains, whereas pure water maintained normal background revertant counts, validating the assay performance. Since a two-fold increase in revertant colonies relative to the vehicle control is generally accepted as evidence of mutagenicity **[12]**, the absence of such a response provides strong evidence that BP does not possess detectable mutagenic activity under the present experimental conditions. The present findings are consistent with previous studies showing the favourable biological characteristics of collagen-based biomaterials used in periodontal regeneration and tissue engineering. Various biomaterials, including collagen and polyglycolic acid (PGA), have showed promising regenerative potential in tissue engineering, gene therapy and neo-organ formation **[13]**. As a collagen-rich biomaterial, bovine pericardium possesses desirable mechanical characteristics, including tensile strength, flexibility and structural stability, while cleaning, solvent dehydration and gamma irradiation contribute to preserving its integrity and suitability for connective tissue remodelling **[14].** In periodontal therapy, resorbable membranes composed of collagen, PLA, PGA and related copolymers are widely used because they eliminate the need for secondary surgical removal while providing an effective barrier for periodontal regeneration **[15].** Previous investigations have showed that bovine pericardium-derived collagen inhibits epithelial migration, promotes connective tissue attachment and maintains structural integrity within periodontal defects, supporting its application as a regenerative membrane **[16].** Furthermore, bovine pericardium has been proposed as both a barrier membrane and scaffold material for guided tissue regeneration **[4],** while clinical studies have showed its favourable performance as a bioabsorbable membrane for periodontal tissue regeneration **[21-23].** Previous studies reported no mutagenic effects of bovine pericardium in the Ames test with S9 metabolic activation **[8]**. The present study extends these findings by evaluating mutagenicity both in the presence and absence of S9 activation, providing additional evidence supporting the genetic safety of bovine pericardium-derived GTR membranes. Similarly, reviews of bovine pericardium membranes used in guided bone regeneration have highlighted their favourable barrier properties and potential advantages over conventional collagen membranes, particularly in implantology **[17].** Additional Ames test investigations performed under comparable experimental conditions have likewise reported the absence of significant mutagenic activity, providing further support for the biological safety of bovine pericardium-derived membranes **[18].** Collectively, these findings reinforce the growing body of evidence supporting the use of bovine pericardium as a safe and biologically compatible regenerative biomaterial. The favourable genotoxicity profile observed in this study may be attributed to the intrinsic biocompatibility of collagen together with the membrane processing procedures, including cleaning, solvent dehydration and gamma irradiation sterilization, which may reduce potentially harmful contaminants while preserving the structural characteristics required for clinical application. Nevertheless, the molecular mechanisms responsible for the absence of mutagenicity remain incompletely understood and warrant further investigation. Despite these encouraging findings, several limitations should be acknowledged. The Ames assay primarily detects gene mutations in bacterial systems and therefore cannot fully characterize genotoxic responses in mammalian cells. Consequently, negative findings in this assay do not completely exclude the possibility of genotoxic effects in higher biological systems. Future investigations should incorporate complementary mammalian genotoxicity assays, including the micronucleus and chromosomal aberration tests, together with *in vivo* studies and long-term biocompatibility evaluations. Comparative studies involving different bovine pericardium membranes and alternative regenerative biomaterials would further strengthen the evidence supporting their biological safety, clinical performance and suitability for guided tissue regeneration.

Conclusion:

We report the mutagenic potential of bovine pericardial membranes in guided tissue regeneration. Ames test showed no mutagenicity, with revertant counts not exceedingly twice the negative control in the presence and absence of S9 activation as reported earlier by us **[8]**. Thus, bovine pericardial membranes are safe for medical use. However, further research is needed to confirm their suitability across diverse applications.

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