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DEVELOPMENT, CHARACTERIZATION AND OPTIMIZATION OF GENTAMYCINSULFATE LOADED BONE GRAFT

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ABSTRACT

Bone defects occur in a wide variety of clinical situations and their reconstruction to provide mechanical integrity to the skeleton is a necessary step in the patient's rehabilitation. The different approaches for bone reconstruction by autografts (transplanted directly from one area of the individual skeleton) and allografts (comes from a donor) has some disadvantages such as the need for a second surgery and the limited quantity and shape of available bone and chances of disease transmission. Here gentamicin sulfate loaded bone grafting material was prepared using dissolution method. The formulation was optimized using Box – Behnken design keeping chitosan, citric acid, and HPMC levels as independent variables. The responses obtained from the design matrix i.e. percentage porosity and mechanical strength were statistically evaluated and an optimum formula was suggested by the design expert software. The setting time of the prepared material was also determined and this parameter was inversely proportional to the number of polymers and the amount of citric acid. The invitro antibacterial study was done using *Staphylococus aureus* by well method and formulation has good zone of inhibition.

KEYWORDS: Gentamycin, Bone grafts, Box-Behnken, Bone substitute.

INTRODUCTION

Infected bone defects

The typical causes of large IBDs are high energy traumas such as explosions, car accidents, or gunshot wounds. Because of this a large number of IBDs are seen in military conflicts. Approximately 80% of injuries sustained during Operations Enduring Freedom and Iraqi Freedom are the result of explosions and 50-85% of these become infected. The majority of these injuries are sustained in the extremities where less protective equipment is worn. In a

prospective study on the treatment of lower extremity open fractures, the outcomes of treatment with either soap or antibiotic solution during irrigation were compared to the outcomes in terms of infection and the healing of the soft and hard tissue.

Bone grafting

Treatment of delayed union, malunion, and non-union is a challenge to the orthopaedic surgeons in veterinary and human fields. Apart from restoration of alignment and stable fixation, in many cases adjunctive measures such as bone-grafting or use of bone-graft substitutes are of paramount importance. Bone-graft materials usually have one or more components: an osteoconductive matrix, which acts as scaffold to new bone growth; osteoinductive proteins, which support mitogenesis of undifferentiated cells; and osteogenic cells, which are capable of forming bone in the appropriate environment. Autologous bone remains the "gold standard" for stimulating bone repair and regeneration, but its availability may be limited and the procedure to harvest the material is associated with complications. Bone-graft substitutes can either substitute autologous bone graftor expand an existing amount of autologous bone graft. We review the currently available bone graft and graft substitutes for the novel therapeutic approaches in clinical setting of orthopaedic surgery.

Harvested bone grafts and graft substitutes: Bone grafts, endogenous or exogenous, are often essential to provide support, fill voids, and enhance biologic repair of skeletal defects due to traumatic or non-traumatic origin. Limitations of use of endogenous bone substance involve additional surgery; often resulting donor site morbidity and limited availability, whereas allograft have been encountered with risk of disease transmission, immunogenicity etc. Therefore, there isa growing need for synthesis of allograft bone substitutes used alone or in combination with other materials (e.g., Allogro [AlloSource, Centennial, Colo], Opteform [Exactech, Inc, Gainesville, Fla].

Calcium phosphate cement: Calcium phosphate ceramics introduced more than three decades ago are considered as bioactive bone substitutes. The paste or injectable calcium phosphates cement offers the advantage of being freely mouldable and adaptable to bone defects. Brown and Chow first reported the formation of apatitic cement consisting of a mixture of tetracalcium phosphate (TetCP) and dicalcium phosphate anhydrite (DCPA). Gruninger et al introduced the term "calcium phosphate cements (CPC)" and described as: 'a powder or as a mixture of powderswhich, upon mixing with water or an aqueous solution to a paste, reacts around room or body temperature by the formation of a precipitate containing

crystals of one or more calcium phosphates and sets by the entanglement of the crystals of that precipitate'. After implantation, this composition form HAp in situ in contact with the physiological fluid. Since its inception CPCs have attracted much attention and different formulations have been put forward. The drawback in using these materials was that close proximity to the host bone was necessary to achieveosteoconduction.

Hardening mechanism

The setting reaction that gives rise to the solid consists in three stages: dissolution of the reactants, nucleation of the new phase (either apatite or brushite) and crystal growth. Therefore, the setting reaction is a dissolution–precipitation process. During dissolution, the raw powders releasecalcium and phosphate ions, generating a supersaturation in the solution. Once the ionic concentration reaches a critical value, the nucleation of the new phase occurs, generally surrounding the powder particles. Afterwards, the new phase keeps growing as the dissolution of the reagents goes on. During the first hours the setting process is controlled by the dissolution kinetics of the raw materials, but once the new phase surrounds the reactants, the process is controlled by diffusion across the new phase.

Bioactivity and Resorption of calcium phosphate cements

One of the most important properties of CPCs is bioactivity. When referring to bone substitutes, abioactive material is one that is able to bind directly with the surrounding bone without the formation of fibrous tissue. Bioactivity, together with the perfect adaptability of the cement pasteduring implantation, leads to a stable connection between defect and implant, speeding up bone healing process. Once implanted, CPCs can be resorbed by two different mechanisms. Active resorption regulated by living cells like macrophages or osteoclasts, and/or passive resorption via chemical dissolution or hydrolysis in the body fluids. Since brushite is soluble in body fluids, brushite cements are mainly resorbed by passive mechanism while appetites being less soluble, cause apatite cements to be mostly resorbed by the active mechanism, i.e. macrophages and osteoclasts locally drop down the pH at values at which apatite becomes soluble. The incorporation of ionic substitutions is a method to control the degradation of CPCs. For example, incorporation of carbonates increases apatite lattice disorder favoring crystal dissolution

Interactions between calcium phosphate Cement and Drugs

The setting reaction of the CPC can be influenced by the presence of the drug either in the liquid or in the powder phase, affecting the final features of the material. Thus, the addition of

a drug can modify the setting kinetics, the rheological properties and the microstructural development of CPCs, all of them relevant for clinical performance.

The impact of drug incorporation on the mechanical properties of CPCs is especially relevant, since it can limit their final applications. Nevertheless, there is no general rule for predicting the effect of drug incorporation in the physicochemical properties of CPCs and more particular in the mechanical properties, because the nature and extent of interaction will depend on the chemical nature of the drug molecule. For instance, some molecules interact with calcium and phosphate ions in solution, inducing a coprecipitation during setting or complexing Ca^{2+}, which results in a delay in the precipitation of the final product and modifies the viscosity of the paste, the setting time and the mechanical properties of the set cement.

MATERIALS AND METHODS

The drug (Gentamycin sulfate) and the other excipients used for the study is purchased from Yarrow chemicals, Mumbai.

Formulation of bone grafting material

Liquid phase: Chitosan, citric acid and HPMC are mixed together in appropriate ratio with distilledwater.

Solid phase: Tri calcium phosphate powder

The solid and liquid components were mixed manually with a spatula for 45 s or around 1 minuteuntil a chewing gum like slurry was achieved. Addition of the mixture in to the molds and allow self-setting. The slurry was placed in to appropriate molds and allow self-setting.

Optimization by box behnken design

Response surface methodology using Box – Behnken was chosen for the optimization of bone grafting material because it allows the determination of influence of the factors with a minimum number of experiments. The independent factors were Chitosan concentration (X1), citric acid (X2) and HPMC (X3). The response variables were Porosity (%) (Y1) and mechanical strength (kg/cm^3) (Y2). seventeen formulations were prepared according to Box - Behnken design.The formulations were F1 to F17. Formulation F13, F14, F15, F16, and F17 were fixed as the central formulation.

Factors	Levels used		
	-1	0	1
X1= Chitosan	1	1.5	2
X2=citric acid	20	30	40
X3=HPMC	0	2	4
Responses	Constraints		
Y1=Porosity	74-90%		
Y2=mechanical strength	5-7kg/cm^3		

Development of the optimum batch

Based on the statistical evaluations the software suggested one optimum batch. The formula for the optimum batch was given in the table no: 2.

Chitosan (%wt)	Citric acid (%wt)	HPMC (%wt)
2	40	4

Evaluation of bone grafting material

1. Injectability

Injectability is the ability to be extruded through a small hole of a long needle. The injectability of Calcium Phosphate Cement is of crucial importance for surgical procedures utilizing minimally invasive procedures such as in vertebroplasty and kyphoplasty or for delivery of the cement in to a very narrow space as in root canal obturation.

It is measured by the weight percentage of the cement paste that could be injected without diminished from a standard syringe by either a hand or a force of 100N maximum. The injectabilitycan be determined by taking 4g of the mixed paste added in to the syringe had a capacity of 5 ml with an opening nozzle size of 2mm in diameter. The paste was extruded after 5 min from the syringe by hand until it was unable to inject entirely. The weight of the injected paste was then measured and injectability was calculated using the following equation.

$$\% \text{ injectability} = \frac{\text{Paste weight expelled syringe}}{\text{Total paste weight before injecting}} \text{ X } 100$$

2. Setting measurement

The setting time was measured by Gilmore needle method. The cement is kept at 37^{0}C was considered set. When a 400 g mass loaded to a needle with a tip diameter of 1mm failed to

makea perceptible circular indentation of the surface of the cement.

3. Determination of mechanical strength

The cement was molded in to definite shape and size. The comprehensive strength was measuredby using Pfizer hardness tester. The mechanical strength was expressed in kg/cm^3.

4. Determination of porosity

The porosity of the scaffolds at different concentrations can be determined by Archimedes' principle. Ethanol was selected as the displacement liquid as it permeates the scaffolds without swelling or shrinking the matrix. The dry weight of scaffolds was denoted as *Wd*, while *Wl* denoted the weight of the scaffold after immersed in the ethanol for 5 min. Then, the scaffolds were removed and the liquid on the surface removed by filter paper. The weight of the wet scaffold was denoted as *Ww*. The porosity of the scaffold was calculated using equation.

$$\text{Porosity (\%)} = \frac{Ww - Wd}{Ww - Wl} \times 100$$

5. Swelling ability

Swelling ability was determined by the percentage of water absorption. Dry weight of the scaffold was denoted as *W0*. Then, porous scaffolds were immersed in PBS buffer solution with pH 7.4 at 37°C for 24 hours. Afterward, the scaffolds were taken out from phosphate buffer solution and itswet weight was measured, denoted as *Ww*.

6. Antibacterial bioactivity

To test the effectiveness of antibiotic released from the bone grafting material samples, a well method is used. For the study, the material is placed on an agar plate seeded with *staphylococcusaureus.* The resulting zone of inhibition was measured after incubating for 24 hours, after which azone of inhibition was measured.

RESULT AND DISCUSSION

1. Preparation of bone grafting materials

Seventeen formulations of bone grafting materials of gentamicin were prepared by dissolution – precipitation method. The bone grafts were prepared with chitosan, citric acid, HPMC and tri calcium phosphate in different ratios.

2. Optimization of bone grafting materials

Bone grafting materials with different drug: polymer ratio was prepared and optimized for the bestformulation.

The Model F-value of 13.18 implies the model is significant. There is only a 0.03% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0003 indicatemodel terms are significant. In this case X_1, X_2^2 are significant model terms. The "Lack of Fit F- value" of 2.15 implies the Lack of Fit is not significant relative to the pure error. There is a 23.98% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fitis good Mathematical relationship in the form of polynomial equation for the measured responseswas obtained with the statistical software.

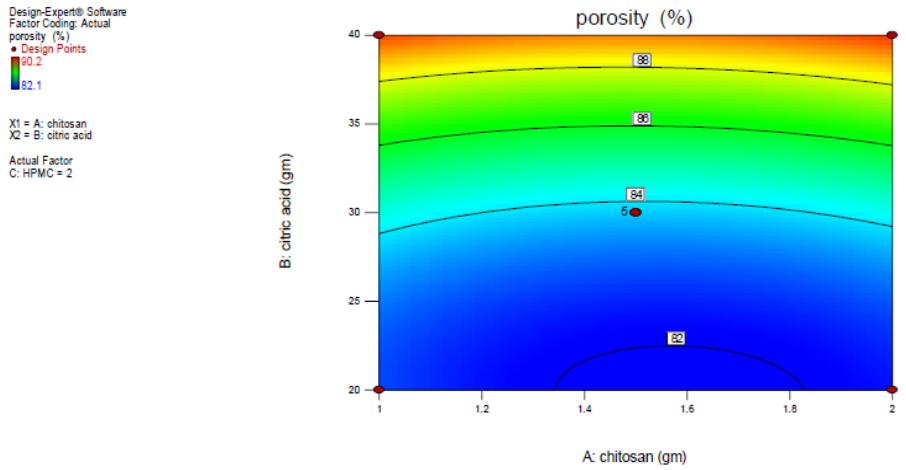

Figure 1: Contour plot for the effect of chitosan, citric acid, HPMC on porosity.

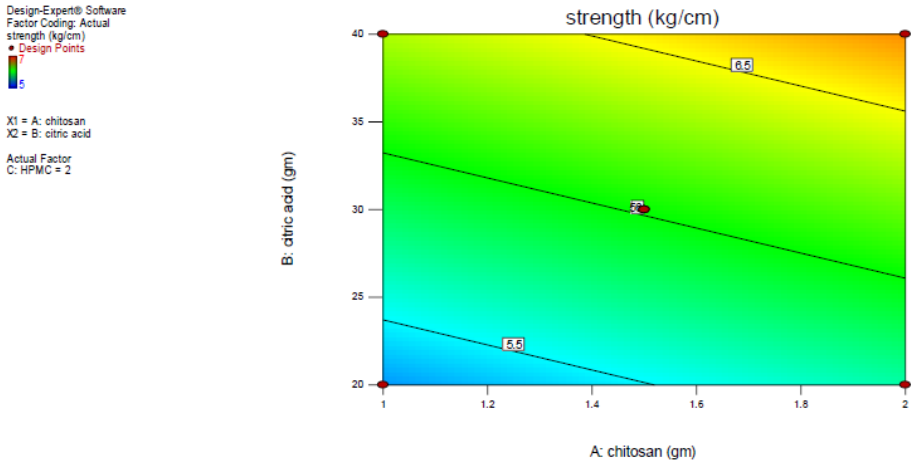

Figure 2: Contour plots for the effect of chitosan, citric acid, HPMC on the mechanical strength.

Development of the optimum BATCH

Table 3: Formula for optimum batch.

Number	Chitosan (%wt)	Citric acid(%wt)	HPMC(%wt)	Porosity (%)	Mechanical strength (kg/cm^3)
1	2	40	4	89.5	6.8

Evaluation of bone grafting material

1. Injectability

Injectability is the ability to be extruded through a small hole of a long needle. It is measured by the weight percentage of the cement paste that could be injected without diminished from a standard syringe by either a hand or a force of 100N maximum.

2. Setting time measurement

The cements were hardened through two processes. The initial process was hardening through thehydration of the salts in the powder component or chelate reactions between one or moreconstituents in the liquid component and powder component. In terms of HPMC content the setting time decreased while the HPMC content increased from 0 to 4%. The powder particles which absorbed citric acid molecules, conserved the amount of water molecules for hydration andincrease the surface are available for dissolution. Hence, the precipitation of the powder component in the liquid component occurred earlier, which decreased the setting time. This mechanism readily explained the results attained in this study. The setting time of F1were shorterthan those of F4 at a similar HPMC content.

3. Mechanical strength

The strength of the cement was tested using Pfizer hardness tester and it was expressed in $kg/cm^{3.}$ The mechanical strength of the cement was dependent on the amount of citric acid and HPMC used in the preparation of cement. Reduced porosity was previously used as a method to improvethe mechanical properties. Therefore, as the citric acid concentration was increased so were the mechanical properties. This was the reason why the mechanical strength of F1 F3 and F11 was better than that of other formulations. In the regards to the mechanical strength of the above formulations in terms of HPMC content, the strength value of F1 was always higher than the HPMC concentration as increased the mechanical strength of the cement was also increased.

4. Determination of porosity

The morphology of calcium phosphate cement may mimic the structure of natural bones which have fully inter connected porous structure which allows cell penetration. The result showed thatwhen the chitosan concentration increased, porosity decreased. For bone grafting, a porous material should have low bio degradability to allow bone regeneration while the ideal porosity should be 85.5% to provide the optimal balance between a better surface area for cell attachment and its structural strength.

5. Swelling ability

Swelling ability may be defined as the ability of a matrix to swells. The swelling ability was determined by the percentage of water absorption. The results showed that the swelling ability of the cement dependent on the chitosan concentration. The swelling ability of the cement was proportional to the chitosan concentration.

6. Anti-bacterial activity

The results of the antimicrobial studies of the formulated gentamicin sulfate loaded bone grafting material against *Staphylococcus.aureus* using well method and the diameter of zone of inhibition. The formulation showed excellent zone of inhibition after 1day incubation at 37^{o}C. Davis and Stout have classified the strength of antibacterial activity in well method based on the zone of inhibition. If the zone of inhibition less than 0.5 cm, the inhibition activity is weak. If the zone of inhibition is 0.5-1 cm, the inhibition activity is moderate. Antibacterial activity of the formulation against *S. aureus* in 1day observation period was concluded as excellent.

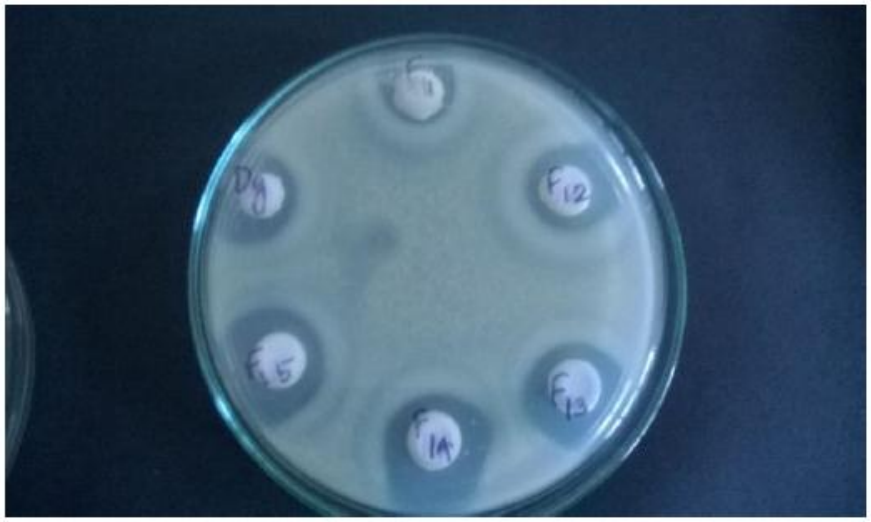

Figure 3: Anti bacterial Activity of gentamicin sulfate bone grafting material against.

Staphylococcus aureus.

Table 4: Result of antibacterial study.

Sl. no.	Organism	Formulation	Zone of inhibition
1	*Staphylococcus aureus*	Gentamicin loaded bone grafting material	2
2	*Staphylococcus aureus*	Pure gentamicin material	2.2

CONCLUSION

Recently calcium phosphate cements have gained much attention worldwide since they show not only biocompatibility but also excellent self-setting ability and moldability. In addition, the morphology of CPC may mimic the structure of natural bones which have fully interconnected porous structure which allows cell penetration. For this reason, CPC have attracted significant interest. As a bone substitute to using real bone many synthetic materials are available as a safe and synthetic also have advantage of not requiring a second procedure to harvest bone, reduces risk and pain. Here gentamicin sulfate loaded bone grafting material was prepared using dissolution method. The responses obtained from the design matrix i.e. percentage porosity and mechanical strength were statistically evaluated and an optimum formula was suggested by the design expert software. The invitro antibacterial study was done using *Staphylococus aureus* by well method and formulation has good zone of inhibition.