

RESEARCH ARTICLE

Controlled release of injectable liposomal *in situ* gel loaded with recombinant human bone morphogenetic protein-2 for the repair of alveolar bone clefts in rabbits

Ali H. Hassan¹, Khaled M. Hosny^{2,3}, Zuahir A. Murshid¹, Adel Alhadlaq⁴, Ahmed Yamani⁵, Ghada Naguib⁶, Hala M. Alkhalidi⁷, and Ahmed R. Afify¹

¹Department of Orthodontics, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia, ²Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia, ³Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Beni Suef University, Beni Suef, Egypt, ⁴Department of Pediatric Dentistry and Orthodontics, Collage of Dentistry, King Saud University, Riyadh, Saudi Arabia, ⁵Department of Oral Surgery and ⁶Department of Restorative Dentistry, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia, and ⁷Department of Clinical Pharmacy, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract

Background and objective: The aim of the present study was to develop and examine a new non-invasive injectable graft for the repair of alveolar bone clefts using recombinant human bone morphogenetic protein-2 (rhBMP-2) encapsulated within injectable liposomal *in situ* gel (LIG).

Method: Different liposomal formulations loaded with rhBMP-2 were prepared, and the effects of the preparation methods and lipid content on the efficiency of rhBMP-2 encapsulation within the liposomes were studied. For the preparation of *in situ* gel, deacetylated gellan gum (DGG) was used, and the *in vitro* gelation characteristics of the gel were evaluated. *In vivo* pharmacokinetics and histology were also assessed. Critical size alveolar defects were surgically created in the maxillae of 30 New Zealand rabbits and treated with different injectable formulae, including rhBMP-2 liposomes and *in situ* gel (rhBMP-2-LIG).

Results: The results indicated that the prepared rhBMP-2-LIG prolonged the release and residence time of BMP-2 within rabbits for more than 7 days. Histomorphometric assessment showed 67% trabecular bone filling of the defects treated using this novel formula.

Conclusion: BMP-2-LIG is a promising delivery device for the repair of alveolar bone defects associated with cleft deformities.

Keywords

Alveolar defect, bone morphogenetic proteins, cleft lip and palate, encapsulation, liposomes

History

Received 1 April 2015
Revised 4 June 2015
Accepted 7 June 2015
Published online 7 July 2015

Introduction

Cleft lip and palate (CLP) is the most common congenital deformities worldwide (Burnham et al., 2007; Falcini et al., 1996). These severe birth defects affect facial structures, particularly maxilla–mandibular structures (Evans, 2004). The treatment of patients with CLP represents a serious problem, as amelioration requires a comprehensive multidisciplinary approach involving multiple surgeries and a lengthy orthodontic treatment. An important step in the treatment of CLP involves bone grafting of the associated alveolar defects (Gimbel et al., 2007).

The most commonly used graft material is the bone removed from ileac crest, which has several shortcomings, such as morbidity at the donor site, limited donor tissue, expected resorption and an associated scarring effect due to

the invasiveness of the surgical procedures (Swan & Goodacre, 2006). Several other grafting techniques involve the use of autologous cells for alveolar cleft bone regeneration and osteoinductive proteins (Hibi et al., 2006; Sawada et al., 2009), such as bone morphogenetic proteins (BMPs).

BMPs are members of the transforming growth factor (TGF- β) superfamily, which act as osteoinductive factors that induce the differentiation of osteoblasts from mesenchymal cells (Hassan et al., 2003; Mori et al., 2000). Previous studies have shown the ability of BMPs to induce bone formation in a variety of models having many clinical applications in orthopaedics and oral and maxillofacial surgery (Geiger et al., 2003; Hassan et al., 2010; Herford & Boyne, 2008; Urist et al., 1984). Delivery systems for biosafe growth factors and biodegradable carriers have been used to achieve the slow, controlled release of growth factors at the site of implantation (Herford & Boyne, 2008). Collagen, tricalcium phosphate, lactideglycolide copolymers, ethyleneglycol-lactic acid copolymer and gelatin hydrogels (Saito et al., 2001; Yamamoto et al., 2006; Zegzula et al., 1997) were demonstrated as successful carriers for BMP-2. The above delivery

Address for correspondence: Khaled Mohamed Hosny, Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia. Tel: +966 592722634. Fax: +966 26951696. E-mail: Elswaify2000@yahoo.com

systems are available in solid states and require conventional surgical procedures for insertion into the cleft defect.

Liposomes are vesicles comprising one or more concentric phospholipid (PL) bilayers enclosing aqueous compartments (Lasic, 1993; Xia & Xu, 2005). Liposome diameters range from 20 nm to several micrometres (μm). Liposomes are frequently used as liquid carriers, reflecting the enhanced absorption and distribution of these molecules (Keller, 2001). In addition, these compounds have the advantage of improving the adhesion and absorption into epithelial cells. The activities of liposomes as carriers depend on several factors, such as composition, membrane rigidity, release rate and encapsulation efficiency (Hosny, 2009a; Sulkowski et al., 2005). Liposomal systems in many instances have shown superior pharmacological properties compared with conventional pharmaceutical products (Foradada & Estelrich, 1995). These materials can be prepared from different types of lipid mixtures (Choudhari et al., 1994), and the physicochemical properties of these compounds can be modified through the modification of the particle size and structure (Nii & Ishii, 2005; Rodriguez et al., 2004).

Recently, *in situ* gelling systems have been used in many medical applications, including controlled drug delivery. Many novel *in situ* implant (ISI)-based matrices have been designed. These formulations are liquid solutions *in vitro*, but convert to gels when in contact with body fluids. These matrices are also considered as mucoadhesive drug delivery systems. The transformation from liquid to gel depends on several factors, according to the type of polymer used, which might convert to gel due to temperature changes, ions present, pH changes or polymer precipitation, leading to controlled drug diffusion and release (Kashyap et al., 2007).

In contrast to stiff gels, *in situ* gels can be readily injected, subsequently swelling and forming a more viscous gel at the site of administration. These gel matrices are also capable of releasing and prolonging the residence time of drugs in a controlled manner (Doijad et al., 2006). *In situ* gels are administered through oral, injectable, rectal, ocular, vaginal and intra-peritoneal routes (Hatefi & Amesden, 2002). Gellan gum is a linear, anionic polysaccharide secreted from the bacterium *Pseudomonas elodea*. Deacetylated gellan gum (DGG) is approved in the USA and EU as a gelling, stabilizing and suspending agent in food products. DGG forms strong clear gels at physiological ion concentrations and has been widely investigated for use as an *in situ* gelling agent, which increases the residence time of any delivered drug (Jansson et al., 2005).

Although, liposomes and *in situ* gel systems are considered as good drug delivery systems, these compounds have never been used to deliver rhBMP-2 to induce bone formation. Therefore, developing a non-invasive method of grafting using recombinant human bone morphogenetic protein-2 (rhBMP-2), delivered in an injectable liposomal ISI delivery system that does not require severe surgical intervention, is a desired therapeutic goal. Bone repair of the alveolar defects in cleft patients is a promising concept to counter the growth retarding effects of conventional grafting techniques, which have serious long-term consequences on overall treatment results.

The objective of the present study was to develop and assess a new non-invasive grafting procedure for the repair of surgically generated alveolar bone clefts using rhBMP-2, encapsulated within injectable liposomal *in situ* gel (LIG).

Experimental methods

Materials

rhBMP-2 was purchased from Creative Biomart (New York, NY). Phosphatidylcholine (extracted from chicken egg yolk), lecithin, cholesterol and DGG were purchased from Sigma Chemical Company (St. Louis, MO). Other chemicals and reagents were of analytical grade and used without further purification.

Preparation and characterization of rhBMP-2 liposomes

Preparation of multilamellar BMP-2 liposome

MLVs containing rhBMP-2 were prepared using the lipid film hydration technique (Szoka & Papahadjopoulos, 1978). Fifty milligrams of lipid components of the liposome matrix (phosphatidylcholine:cholesterol at different ratios, including 5:1, 5:2, 5:3, 5:4 and 5:5) were solubilized in chloroform (10 ml) in a round-bottomed flask. The organic solvent was evaporated at 33 °C on a rotary evaporator to form a thin lipid film. Liposomes were prepared by dispersing the lipid component film of the various liposomal formulations in an isotonic phosphate buffer saline solution pH 7.4 (10 ml) containing rhBMP-2 (30 μg). Finally, the liposomal suspension was passed 10 times through 2.2- μm nitrocellulose filters (GS, Millipore, Eschborn, Germany) for purification and stored in a refrigerator.

Preparation of REVs liposomes

BMP-2 unilamellar (REVs) liposomes were prepared using the reverse-phase evaporation technique (Gilbert et al., 1987). Fifty milligrams of lipid components of the liposome matrix (phosphatidylcholine:cholesterol at different ratios, including 5:1, 5:2, 5:3, 5:4 and 5:5) were solubilized in a chloroform:methanol mixture (1:2 v/v) in a round-bottomed flask. The organic solvent was evaporated at 40 °C on a rotary evaporator to form a thin lipid film. The lipid film was redissolved in 10 ml ether, and the rhBMP-2 solution (30 μg), in a 20-ml mixture of acetone and phosphate buffered saline (PBS, pH 7.4) (1:1 v/v), was added. The liposomes were equilibrated at room temperature, and 10 ml PBS was subsequently added to the liposomal suspension and passed 10 times through 2.2- μm nitrocellulose filters (GS, Millipore, Eschborn, Germany) for purification and stored in a refrigerator overnight.

Measurement of liposome particle size, and encapsulation efficiency (EE%)

The particle size of the prepared liposomes was measured using a zetasizer (Microtrac, Montgomeryville, PA), and the encapsulation of rhBMP-2 was measured through a dialysis membrane with a molecular cutoff size of 12 000 Da and pore

size of 2.4 nm for dialysis. The dialysis membrane (1×10 cm) was activated after soaking in Millipore-distilled water for 2 h. One end of the membrane was tied with the thread, and then the liposomal suspension was poured into the opened end, which was subsequently tied with a thread. The bag was suspended in a beaker containing 200 ml of distilled water and placed on a magnetic stirrer. The samples were withdrawn from the distilled water at particular time intervals, and the absorbance was measured at 280 nm in a spectrophotometer. The encapsulation efficacy was obtained as the mass ratio between the amount of BMP-2 incorporated in the liposomes and that used in the liposome preparation.

Preparation and characterization of rhBMP-2 LIG

Formulation of *in situ* gel

The *in situ* gel was prepared by sprinkling (50, 100, 150 and 200 mg) DGG over 6 ml distilled water at $80 \pm 1^\circ\text{C}$. The solutions were stirred at 50 rpm until the polymer was dissolved and subsequently cooled overnight (Jansson et al., 2005). A separate 4 ml of rhBMP-2 liposomal dispersion was added to the polymeric solution and mixed. The final *in situ* gelling solutions, containing 0.5%, 1%, 1.5% and 2% DGG, were stored overnight in the refrigerator prior to further evaluation.

Evaluation of *in situ* gels

Critical ionic concentration (CIC) for DGG phase transition

The critical ionic concentration (CIC) for phase transition is an important parameter for ion-activated *in situ* gels. The CIC was determined after mixing 1 ml of DGG solutions with different concentrations and various amounts of artificial body fluid (ABF), comprising 150 ± 32 mM NaCl, 41 ± 18 mM KCl and 4 ± 2 mM CaCl_2 , in bottles placed in a water bath at 32°C . After 20 s, the bottles were turned over. If the gels adhered to the bottom instead of flowing or sliding down the side, then the formulation showed good gel formation and was considered “+”. The CIC was estimated as the minimum ABF volume in 1 ml of DGG mixture required for the induction of gel formation. For instance, mixing 1 ml of the DGG solution with 0.25 ml of ABF produced a gel with a CIC value of 0.25.

Rheological properties of the ISI

The apparent single viscosity values were measured using a Brookfield digital viscometer. The viscosity of the rhBMP-2 LIG was measured at a controlled rate of 10 rpm (spindle 6) after 30 s before and after gelation.

Measurement of gel strength

A sample of the formulation (5 g) was gelled after mixing the formula with ABF according to the previously measured CIC. A 3.5 g (0.7 cm diameter) weight was placed on top of the gel. The gel strength, as an indication of the viscosity of the *in situ* gel under physiological conditions, was determined based on the time, in seconds, required for the weight to penetrate 3 cm into the gel.

In vitro release study

A 5-ml sample of the liposomal ISI was placed in a dialysis bag and immersed into a vessel containing 100 ml PBS, pH 6.8 at $37 \pm 1^\circ\text{C}$; the solution was stirred at 50 rpm for 120 h. The samples were collected at different time intervals, and the (BMP-2) content was measured using a spectrophotometer at 280 nm. The test was repeated for the control *in situ* gel prepared by dissolving BMP-2 in DGG aqueous solution.

The *in vivo* study

Study design

Critical size alveolar defects were surgically created bilaterally in the maxilla of New Zealand rabbits. This research was approved through the Research Ethics Committee of the Faculty of Dentistry at King Abdulaziz University, Jeddah, Saudi Arabia. A total of 30, 7-week-old, male, New Zealand white rabbits weighing 2.5–3.0 kg were enrolled in this study. The surgical procedure to create experimental alveolar clefts was performed according to the method of Puumanen et al. (2005), with some modifications. After proper sedation and disinfection, full thickness mucosal flaps were reflected to expose buccal maxillary bone, and a $10 \times 5 \times 5$ mm osseous defect was created with a round burr on each side of maxillary bone at approximately 5 mm distal to the incisor teeth. The soft tissue flap was carefully sutured back over the defects and left to heal for 7–10 days. Post-surgically, the wounds were clinically assessed for any potential dehiscence or failure. The rabbits subsequently received the assigned solution through injection. The animals were divided into five groups according to treatment. Group A animals were injected with rhBMP-2 in isotonic solution (positive control), Group B animals were injected with rhBMP-2 liposomal suspension, Group C animals were injected with rhBMP-2 dispersed in the *in situ* gel base, Group D animals were injected with rhBMP-2 LIG and the Group E animals were injected with isotonic saline without rhBMP-2 (negative control). For pharmacokinetic evaluation, the plasma samples were collected from the five groups prior to injection and at 1, 2, 3, 6, 12 and 24 h and 2, 4 and 7 days. All samples were analyzed for rhBMP-2 content using specific ELISA assays.

All animals were administered a standard diet and housed in separate cages in an air-conditioned room at a constant temperature of $22 \pm 1^\circ\text{C}$ and humidity of $50 \pm 5\%$ with a 12 h/12 h light/dark lighting cycle. The general health of the animals was also evaluated daily. After 6 weeks, the rabbits were euthanized using CO_2 according to the regulations of the King Abdul Aziz University Animal Care Committee. The maxillae were dissected, fixed, processed and stained for histological assessment. Histologic sections were assessed under light microscopy. Quantitative histomorphometric analysis was performed to measure the total bone volume in the created defects using digital images captured for five slides per sample at different depths of the defects and standardized to be at the centre of each defect on each slide. The images were captured using a digital camera with $10\times$ magnification lenses (Olympus DP72 camera, Olympus Optical Co., Ltd, Tokyo, Japan). The field of view of each image was standardized using a calibration ruler of 50 μm in length.

Histomorphometric analysis was performed using imageJ 1.48v software (National Institute of Health, Bethesda, MD). Multiple comparisons among the groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test using SPSS statistical package Version 16 (SPSS, Chicago, IL). The level of significance was set at 5%.

Results and discussion

Factors affecting encapsulation efficiency

The results in Table 1 show that the rhBMP-2 encapsulation efficiency varied with the method of preparation and lipid composition used in the preparation of liposomes. The encapsulation efficiency of BMP-2 in liposomes showed that the EE% of liposomes created using the REV technique had higher values in REV than in MLVs of the same composition and molar ratio, as these liposomes were prepared in aqueous phosphate buffer, pH 7.4, and BMP-2 has a higher water solubility than the solubility of organic solvents. Although, multiple lamellae of MLVs are capable of loading a higher mass of lipophilic drug than REV (Perugini & Pavanetto, 1998), the EE% of these liposomes was lower than that of REV of the same composition and molar ratio. The encapsulation efficiency of rhBMP-2 liposomes increased as the cholesterol increased up to a molar ratio of PC:CH of 5:4. The increase in EE% associated with increases in the CH content reflected the fact that the CH increased the rigidity of liposomes and the rigidity of bilayers, resulting in higher stability and reduced permeability of the liposomal membrane (New, 1990), and hence greater drug retention (Gulati et al., 1998). Decreasing EE% with increasing cholesterol ratio above 5:4 might reflect excess cholesterol disrupting the regular linear structure of the liposomal membrane (Hosny, 2009b). Thus, the 5:4 ratio (phospholipid:cholesterol) was the most efficient regarding the EE% (Table 1). In summary, these results indicate that liposomes

prepared as REV with a lipid composition of PC:CH in molar ratio (5:4) showed a higher EE% (87 ± 3) than other liposome formulations. Thus, all subsequently formed liposomes used for *in vitro* and *in vivo* studies were unilamellar reverse-phase types.

Formulation and evaluation of the LIG

Each of the ISIs had a clear appearance. The results of the gelation studies, CIC values and viscosities of the ISIs in solution and gel form are given in Table 2. The gel strength values between 25 and 50 s were considered sufficient, and the gels that exhibited gel strengths within this range were selected for *in vitro* release.

Based on these results, it is clear that ISIs were successfully prepared using 0.5–2% DGG as an ion-induced gelling agent. All of the LIGs mixtures had a clear appearance. The results from the viscosity tests for the ISIs in solution and gel form indicated a marked increase in viscosity values (16.02–45.01 cp) in the presence of BF compared with the LIGs in solution (2.62–4.81 cp), and this change solely reflected the gel conversion, which indicated the *in situ* nature of the prepared implants.

When developing an injectable LIG, the gel strength is an important factor to consider. The ideal gel strength facilitates the easy injection of solution without leakage from the injection site. It is important that the gel formulation has suitable gel strength. Gel strength less than 25 s might not have sufficient structural integrity and might flow away from the injection site. However, gels with strength greater than 50 s might be too stiff and cause discomfort (Hosny & Banjar, 2013). For this reason, the LIG-3 was selected for further evaluation.

Figure 1 shows the *in vitro* release profile of (BMP-2) from the selected LIG-3 formulation and from the control *in situ* gel prepared after dissolving rhBMP-2 in 1.5% DGG solution and gelling with ABF. The *in vitro* release profiles for LIG-3

Table 1. Particle size and encapsulation efficiency of BMP-2 in multilamellar and reversed-phase evaporation liposomes.

Liposomal lipid composition	Molar ratio	MLVs particle size (μm)	MLVs encapsulation efficiency ± SD	REVs particle size (μm)	REVs encapsulation efficiency ± SD
PC:CH	5:1	1.231	15.13 ± 0.44^a	0.847	54.13 ± 2.13^d
PC:CH	5:2	1.423	29.14 ± 2.22^b	0.931	61.31 ± 3.35^e
PC:CH	5:3	1.539	33.18 ± 2.57^b	1.121	75.21 ± 3.68^f
PC:CH	5:4	1.623	39.25 ± 3.22^c	1.243	87.11 ± 3.48^g
PC:CH	5:5	1.417	37.36 ± 2.97^c	1.143	80.23 ± 3.65^g

Groups identified using different superscript letters are significantly different at $p < 0.05$.

PC indicates egg phosphatidylcholine; CH, cholesterol.

Each value given in the table was calculated from $n = 3$ parallels, indicating the means ± SD.

Table 2. CIC%, gelling capacity, gel strength and viscosities ± SD in solution and gel form for different prepared LIGs.

Groups	Formulation	DGG (mg)	CIC (%)	Gelling capacity	Viscosity before gellation (cp)	Viscosity after gellation (cp)	Gel strength (s)
A	LIG-1	50	25	+	2.62 ± 0.21	16.02 ± 1.15	14.12
B	LIG-2	100	20	++	2.81 ± 0.28	21.25 ± 2.35	23.65
C	LIG-3	150	17	+++	3.11 ± 0.29	32.14 ± 4.11	38.28
D	LIG-4	200	13	+++	4.81 ± 0.40	45.01 ± 4.22	56.36

ISI – *in situ* implant; DGG – deacetylated gellan gum; CIC – critical ionic concentration of ABF; cp – centipoises.

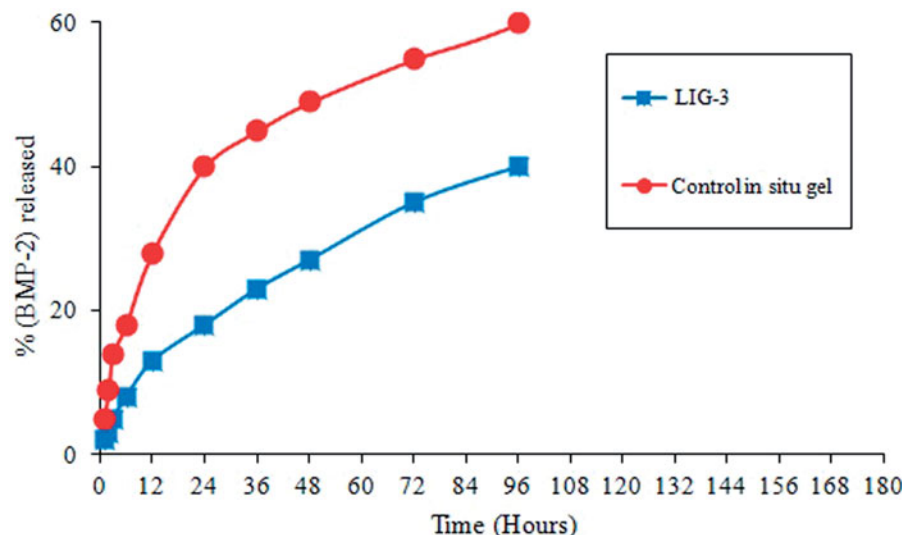


Figure 1. *In vitro* (BMP-2) release profile from LIG formulations (LIG-3) and control *in situ* gel.

and control gel exhibited inflection points after 12 h in the case of LIG-3 and after 24 h in the case of control *in situ* gel. The initial release rate of the drug was rapid, reflecting incomplete gel formation, but the release rapidly decreased after the gel was completely formed and remained at a steady state.

The results for the control *in situ* gel showed a much faster release of rhBMP-2 during the first 12 h (26% release of injected dose), compared with LIG-3, which only released 7% of the injected dose after 12 h, indicating that the encapsulation of rhBMP-2 within the REVs prior to incorporation within the *in situ* gel base significantly affects the release profile of rhBMP-2 and controls the release of this compound from the LIG during the test period. The slower transport across the membrane from the liposome system compared with free BMP implies a reduced driving force for transfer across the dialysis tubing, which might also indicate slow release from these liposomes.

The *in vivo* studies

Histological and histomorphometrical assessments showed significantly different amounts of bone formation between the different groups, with the highest total bone volume measured in Group D (Table 3). In addition, the results of the *in vivo* drug release study (Figure 2) after injection were consistent with the histological results.

After healing for 10 days, the rabbits were injected with LIG-3 containing 5 µg/kg rhBMP-2. Blood samples were collected at 12, 18, 24, 48, 72 and 96 h. Considering the plasma levels of rhBMP-2, following observations were made:

The results of ELISA showed that in Group A, after injection with rhBMP-2 dissolved in isotonic saline solution, the levels of BMP-2 rapidly increased during the first 12 h to more than 20 µg/l and subsequently decreased to less than 2 µg/l within 48 h (Figure 2). This result is consistent with the histological examination, revealing that there were few bony spicules embedded in a fibrous tissue filling the defect. The histomorphometric analysis revealed that the repaired defects

Table 3. Comparison of the total bone volume measured at the created bone defects using one-way ANOVA.

Group	Gel	rhBMP-2 (µg/kg)	Liposomes	96 h [BMP-2] (µg)	Bone formation
A	—	5	—	<2	19 ± 3% ^a
B	—	5	+	2	35 ± 6% ^b
C	+	5	—	<1	27 ± 1% ^b
D	+	5	+	4	67 ± 12% ^c
E	—	5	—	0	12 ± 4% ^a

Groups identified with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA and Tukey's multiple comparisons.

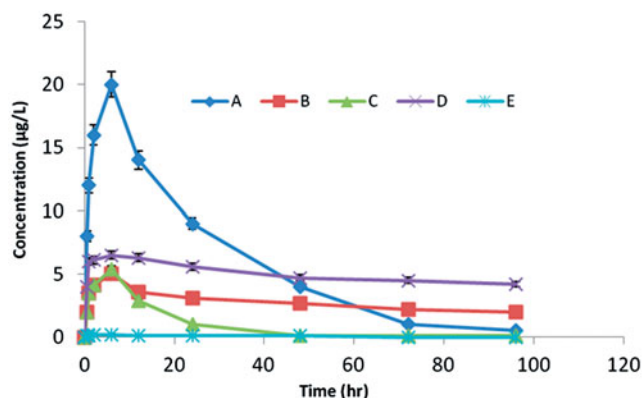


Figure 2. Mean plasma levels of BMP-2 after the local injection of 5 µg/kg of BMP-2 isotonic saline into defects in rabbits (A), BMP-2 in unilamellar liposomal suspension (B), BMP-2 dispersed in the *in situ* gel (C), rhBMP-2-LIG-3 formula (D) and negative control (E).

observed in Group A were on average 19% (SD, 3) ossified (Table 3). This observation reflects the fact that BMP-2 was dissolved in isotonic saline only without any gelling agent, consistent with many previous studies showing that the BMP-2 is inefficient in the absence of a controlled release delivery system (Haidar et al., 2009).

In Group B, the plasma level of BMP-2 rapidly increased to 5.5 µg/l within 12 h (Figure 2). After 12 h, the plasma level declined, slowing to a rate of less than 3 µg/l within 100 h.

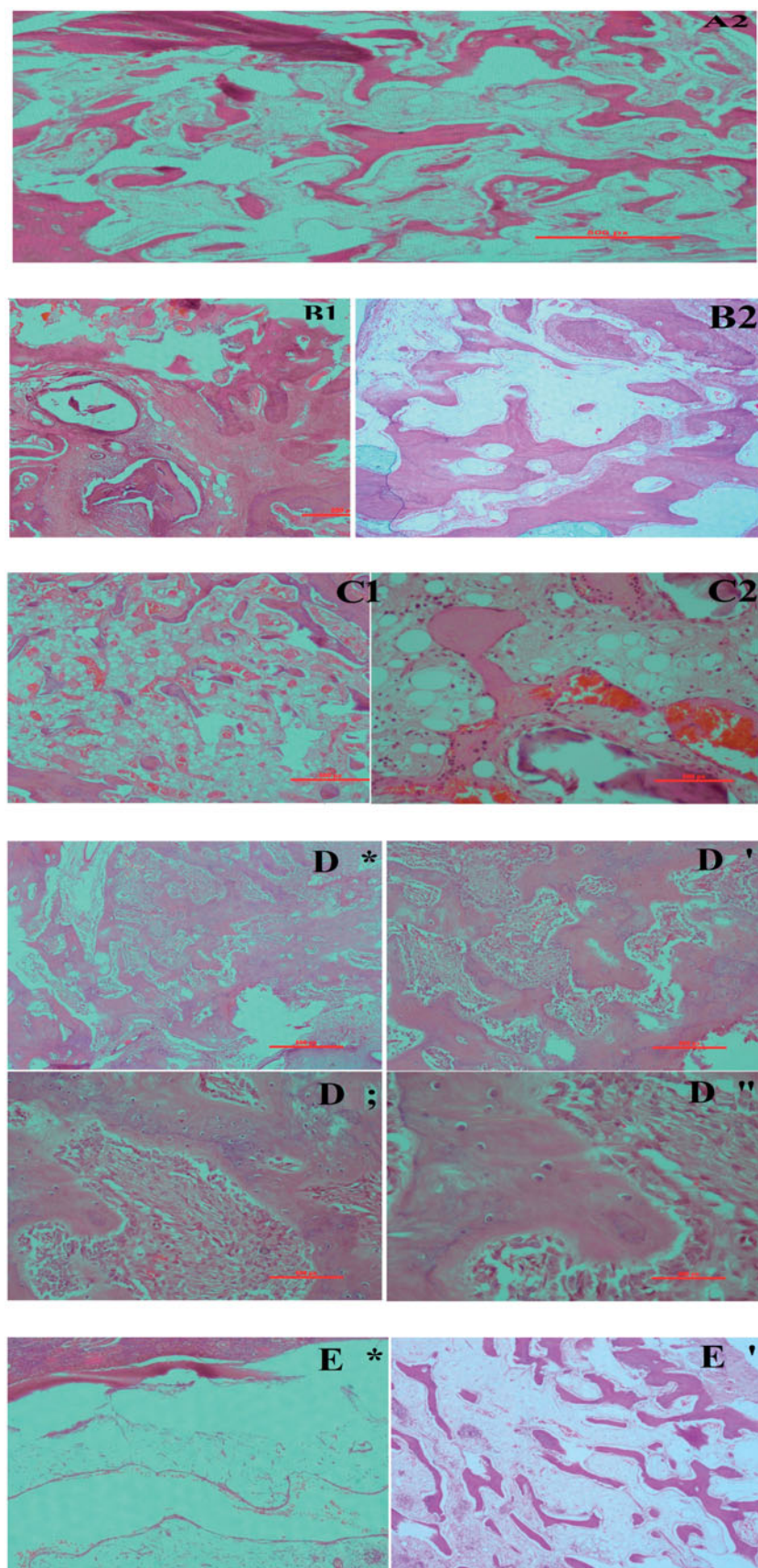


Figure 3. Photomicrograph of a bone defect in Group A, Group B (slides stained with H&E, at $4\times$ (B^1) and $40\times$ (B^2)), Group C (slides stained with H&E, at $4\times$ (C^1) and $40\times$ (C^2)), Group D (slides stained with H&E, at $4\times$ (D^* ,') and $40\times$ (D ;',')) and Group E (slides stained with H&E, at $4\times$ (E^*) and $10\times$ (E')).

The elevated plasma level of rhBMP-2 over the 100-h period reflected the encapsulation of rhBMP-2 within liposomes, which retarded the release of the BMP-2 and maintained a higher plasma rhBMP-2 concentration after 100 h. This result is consistent with the histological examination (Figure 3). The repaired defects in Group B were on average 35% (SD, 1) ossified (Table 3). Islands of mature bone entrapped within fibrous tissue were also observed, with no evidence of any remaining liposomes.

In Group C, in which defects were repaired after injecting rhBMP-2 dissolved in the *in situ* gel polymeric solution without liposomes, and the plasma level of BMP-2 rapidly increased to more than 5 µg/l within 12 h. After 12 h, the plasma level declined to less than 0.5 µg/l within 48 h (Figure 2). This result reflects the fact that in this formula, BMP-2 is dissolved in an *in situ* polymer without entrapping the protein in any retarding system, consistent with the histological examination, which revealed residual polymer appearing as empty vacuoles entrapped in fibrous tissue filling most of the defect. The total bone volume formed in the defect was on average 27% (SD, 1). The newly formed bone was trabecular in nature with multiple blood vessels within the surrounding fibrous tissue (Figure 3).

In Group D (LIG-3 containing rhBMP-2 encapsulated within REVs liposome and suspended within an *in situ* gel base), the plasma level of rhBMP-2 slowly increased to 5.5 µg/l within 18 h, and after 18 h, the plasma level did not significantly decrease but remained at a steady state during the test period (Figure 2). These experimental variables were optimum for the release of rhBMP-2, reflecting the availability of this compound at the defect, where the polymer was assumed to bind with soft tissue and subsequently release the protein in an optimum manner. Thus, in this formula, the rhBMP-2 is entrapped within liposomes, which retard the release of rhBMP-2, but the liposomes are enveloped within the *in situ* polymer, which maintains the concentration in steady state for a longer period of time, consistent with the histological examination, which revealed active bone formation. Most of the defect was filled with newly formed bone (67% [SD, 12]), with multiple surrounding osteoblasts. The newly formed bone was surrounded with fibro-vascular tissues. Although, the newly formed bone did not fully repair the defect, the presence of fibro-vascular tissue containing a large number of osteoblasts suggests that with time, this defect might be fully repaired (Figure 3).

In Group E (animals injected with isotonic saline without any rhBMP-2 (negative control)), the plasma levels of rhBMP-2 were untraceable during the entire release study (Figure 2), consistent with the histological examination (Figure 3), which revealed few bone spicules in some slides and the complete absence of any newly formed bone in other slides (Table 3), with mostly fibrous tissue filling the entire defect.

In the present study, we provided the first demonstration of a novel injectable method for regenerating alveolar bone without the addition of exogenous bone in growing rabbits. This method could be of great advantage in the early treatment of alveolar defects associated with CLP deformities, which as hypothesized might provide the foundation for a new, simple treatment of alveolar defects, in a minimally invasive manner. Such an approach should help to reduce the

morbidity, risk and retardation effects of the standard grafting techniques. The created defect was designed to have a critical size similar to the alveolar cleft as described by Puumanen et al. However, animal models with natural clefts should be used in future studies to better simulate the actual application in humans and assess the effect of such grafting technique on maxillary growth.

While others have injected BMPs bound to collagen or other resorbable matrices, we encapsulated rhBMP-2 in unilamellar liposomes suspended in a deacylated gellan gum gelling agent. Preliminary *in vitro* studies of the liposomal composition, encapsulation efficiency, gel formation, gel viscosity and gel strength were performed to optimize the composition of liposomes and gels. To our knowledge, this study is the first where rhBMP-2 has been encapsulated in liposomes that were in turn suspended in gelling agents to form stiff gels when exposed to ionic gelation in body fluids. *In vitro* rhBMP-2 release kinetics revealed that the gel provided a more gradual, steady release of rhBMP-2 compared with non-gelled controls. The optimal gel was easily injected as a solution but quickly developed enough stiffness after injection to not leak back through the needle tract.

The pharmacokinetics of rhBMP-2 uptake and plasma profiles over 7 days release showed that liposomes provided some sustained release of rhBMP-2 and the second highest amount of bone formation at the injection site 6 weeks later (Table 3, Group B). However, when liposomes containing rhBMP-2 were suspended in the gelling agent, the plasma levels remained higher (Table 3, Group D) than any of the other group. Group D animals also produced significantly ($p < 0.06$) more bone than any of the other groups.

This animal study was performed in adult rabbits. However, whether even more bone would have formed in younger animals with more stem cells. Histological examination of the injection site did not reveal traces of the liposomes or gels, suggesting that these compounds were completely resorbed in 6 weeks. We attribute the success of this study to the prolonged elevation of rhBMP-2 in the plasma of Group D animals. We speculate that the prolonged elevation of rhBMP-2 facilitates the recruitment and transformation of enough osteoblasts and an adequate level of angiogenesis to heal a critical-sized alveolar defects in 6 weeks.

Conclusion

The controlled release of injectable rhBMP-2 LIG is a novel drug delivery system that provided a new non-invasive procedure to repair alveolar cleft defects. The treated defects were 67% ossified with surrounding fibro-vascular tissues and multiple osteoblasts within 6 weeks. Long-term healing effects of this treatment should be investigated with different doses of rhBMP-2 and longer intervals of testing. Indeed, it will not obviate the need for further clinical evaluation for this novel formula, which might inform clinicians of other important data.

Declaration of interest

This research was funded through the Deanship of Scientific Research (DSR) at King Abdulaziz University (KAU) through

grant number MG/31/4 for the group entitled “Dentofacial deformities and cleft lip and palate”.

The authors declare that there are no conflicts of interest.

References

- Burnham JM, Shults J, Petit MA, et al. (2007). Alterations in proximal femur geometry in children treated with glucocorticoids for Crohn disease or nephrotic syndrome: impact of the underlying disease. *J Bone Miner Res* 22:551–9.
- Choudhari KB, Labhasetwar V, Dorle AK. (1994). Liposomes as a carrier for oral administration of insulin: effect of formulation factors. *J Microencapsul* 11:319–25.
- Doijad RC, Manvi FV, Malleswara Rao V, Alase P. (2006). Sustained ophthalmic delivery of gatifloxacin from in situ gelling system. *Indian J Pharm Sci* 68:814–18.
- Evans CA. (2004). Orthodontic treatment for patients with clefts. *Clin Plast Surg* 31:271–90.
- Falcini F, Trapani S, Civinini R, et al. (1996). The primary role of steroids on the osteoporosis in juvenile rheumatoid patients evaluated by dual energy X-ray absorptiometry. *J Endocrinol Invest* 19:165–9.
- Foradada M, Estelrich J. (1995). Encapsulation of thioguanine in liposomes. *Int J Pharm* 24:261–9.
- Geiger M, Li RH, Friess W. (2003). Collagen sponges for bone regeneration with rhBMP-2. *Adv Drug Deliv Rev* 55:1613–29.
- Gilbert JC, Richardson JL, Davis MC, et al. (1987). The effect of solutes and polymers on the gelation properties of pluronic F-127 solutions for controlled drug delivery. *J Control Release* 5:113–18.
- Gimbel M, Ashley RK, Sisodia M, et al. (2007). Repair of alveolar cleft defects: reduced morbidity with bone marrow stem cells in a resorbable matrix. *J Craniofac Surg* 18:895–901.
- Gulati M, Grover M, Singh M, Singh S. (1998). Study of azathioprine encapsulation into liposomes. *J Microencapsul* 15:485–94.
- Haidar ZS, Hamdy RC, Tabrizian M. (2009). Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part B: delivery systems for BMPs in orthopaedic and craniofacial tissue engineering. *Biotechnol Lett* 31:1825–35.
- Hassan AH, Al-Hubail A, Al-Fraidi AA. (2010). Bone inductive proteins to enhance postorthodontic stability. *Angle Orthod* 80:1051–60.
- Hassan AH, Evans CA, Zaki AM, George A. (2003). Use of bone morphogenetic protein-2 and dentin matrix protein-1 to enhance the osteointegration of the Onplant system. *Connect Tissue Res* 44:30–41.
- Hatefi A, Amesden B. (2002). Biodegradable injectable in-situ forming drug delivery systems. *J Control Release* 80:9–28.
- Herford AS, Boyne P J. (2008). Reconstruction of mandibular continuity defects with bone morphogenetic protein-2 (rhBMP-2). *J Oral Maxillofac Surg* 66:6166–75.
- Hibi H, Yamada Y, Ueda M, Endo Y. (2006). Alveolar cleft osteoplasty using tissue-engineered osteogenic material. *Int J Oral Maxillofac Surg* 35:551–5.
- Hosny KM. (2009a). Preparation and evaluation of thermosensitive liposomal hydrogel for enhanced transcorneal permeation of ofloxacin. *AAPS PharmSciTech* 10:1336–42.
- Hosny KM. (2009b). Ciprofloxacin as ocular liposomal hydrogel. *AAPS Pharmascitech* 2:241–6.
- Hosny KM, Banjar ZM. (2013). The formulation of a nasal nanoemulsion zaleplon in situ gel for the treatment of insomnia. *Expert Opin Drug Deliv* 10:1033–41.
- Jansson B, Hägerström H, Fransén N, et al. (2005). The influence of gellan gum on the transfer of fluorescein dextran across rat nasal epithelium in vivo. *Eur J Pharm Biopharm* 59:557–64.
- Kashyap N, Viswanad B, Sharma G, et al. (2007). Design and evaluation of biodegradable, biosensitive in situ gelling systems for pulsatile delivery of insulin. *Biomaterials* 28:2051–60.
- Keller BC. (2001). Liposomes in nutrition. *Trends Food Sci Technol* 12: 25–31.
- Lasic DD, ed. (1993). Preparation of liposomes. In: *Liposomes: from physics to application*. Amsterdam, The Netherlands: Elsevier Science Publisher B.V.; 1993:63–107.
- Mori M, Isobe M, Yamazaki Y, et al. (2000). Restoration of segmental bone defects in rabbit radius by biodegradable capsules containing recombinant human bone morphogenetic protein-2. *J Biomed Mater Res* 50:191–8.
- New RC. (1990). *Liposomes: a practical approach*. Oxford: Oxford University Press.
- Nii T, Ishii F. (2005). Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. *Int J Pharm* 298:198–205.
- Perugini P, Pavanetto F. (1998). Liposomes containing boronophenylalanine for boron neutron capture therapy. *J Microencapsul* 15:473–83.
- Puumanen K, Kellomäki M, Ritsilä V, et al. (2005). A novel bioabsorbable composite membrane of polyactive 70/30 and bioactive glass number 13–93 in repair of experimental maxillary alveolar cleft defects. *J Biomed Mater Res B Appl Biomater* 75:25–33.
- Rodriguez S, Allémann E, Fessi H, Doelker E. (2004). Physicochemical parameters associated with nanoparticle formation in the salting-out, emulsification-diffusion, and nanoprecipitation methods. *Pharm Res* 21:1428–39.
- Saito N, Okada T, Horiuchi H, et al. (2001). A biodegradable polymer as a cytokine delivery system for inducing bone formation. *Nat Biotechnol* 19:332–5.
- Sawada Y, Hokugo A, Yangm Y, et al. (2009). A trial of alveolar cleft bone regeneration by controlled release of bone morphogenetic protein: an experimental study in rabbits. *Oral Maxillofac Surg* 108: 812–20.
- Sulkowski WW, Pentak D, Nowak K, Sułkowska A. (2005). The influence of temperature, cholesterol content and pH on liposome stability. *J Mol Struct* 744:737–47.
- Swan MC, Goodacre TE. (2006). Morbidity at the iliac crest donor site following bone grafting of the cleft alveolus. *Br J Oral Maxillofac Surg* 44:129–33.
- Szoka F, Papahadjopoulos D. (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci USA* 75:4194–8.
- Urist MR, Lietze A, Dawson E. (1984). Beta-tricalcium phosphate delivery system for bone morphogenetic protein. *Clin Orthop Relat Res* 177:277–80.
- Xia SQ, Xu SY. (2005). Ferrous sulfate liposomes: preparation, stability and application in fluid milk. *Food Res Int* 38:289–96.
- Yamamoto M, Takahashi Y, Tabata Y. (2006). Enhanced bone regeneration at a segmental bone defect by controlled release of bone morphogenetic protein-2 from a biodegradable hydrogel. *Tissue Eng* 12:1305–11.
- Zegzula HD, Buck DC, Brekke J, et al. (1997). Bone formation with use of rhBMP-2 (recombinant human bone morphogenetic protein-2). *J Bone Joint Surg Am* 79:1778–90.