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Chitosan and alginate scaffolds for bone tissue regeneration

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Polymeric scaffold for tissue regeneration was developed for veterinary applications. Oxytetracycline hydrochloride (OTC), which is a widely used antibiotic in veterinary medicine was chosen as the model compound. Gel formulations using chitosan and alginate were prepared in distilled water or in 1% (v/v) acetic acid solution. Sponges were also prepared by a freeze-drying process. Tripolyphosphate was used for cross-linking. Viscosity was decreased in the presence of OTC in chitosan gels whereas no difference was found with alginate gels. All gels showed pseudoplastic behaviour. Water absorption capacity was highest with chitosan/alginate sponges. The solvent used for preparation of the chitosan gels was found to affect the release of OTC. The release of OTC from the sponges was increased by cross-linking. Chitosan/alginate sponges showed the slowest and lowest drug release among the developed sponge formulations in this study. The formulations were found to be biocompatible, inducing no adverse reaction *in vivo* on surgically formed bone defects of radius of rabbits. The level of organisation of the remodelled new bone in the treatment groups was better than that of control. Incorporation of OTC into formulations did not show any considerable enhancing effect.

1. Introduction

Bone fractures or defects are common problems for companion animals. Trauma is the most common primary cause of fractures or defects in small animals and is usually associated with traffic accidents, a fall from a height or fights among animals. Bone defects may also be secondary to a pathology of the bone, such as cystis tumours, infections and metabolic diseases (Sheikh 2000).

Polymeric scaffolds are manufactured for local delivery of active agents to promote bone regeneration, and to prevent bone and wound infection at the application site. The local administration of antibiotics in drug delivery systems in long bone defects decreases the time of bone healing and prevents bone and wound infection at the site (Korkusuz et al. 2001; Vats et al. 2003). Scaffolds based on chitosan and alginate offer advantages over other polymers due to their non-toxicity, biodegradability, biocompatibility, favorable mechanical properties, and are shown to be suitable for cell ingrowth and reconstruction (Alsberg et al. 2001; Lee and Mooney, 2001; Lai et al. 2003; Seol et al. 2004; Şenel and McClure, 2004). Three dimensional and porous structure of gels or sponges can provide a scaffold for bone cells to grow in and expansion of cells regularly to form the new bone tissue and its biodegradability allow the drugs to be released to implantation sites (Drury and Mooney, 2003; Vats et al. 2003). A bioadhesive scaffold exhibits a good retention at the application site and ease of delivery. Bioadhesive gels can fill the surface of the biological substrate due to their penetrating ability to the tissue cavities and increase remaining time of the active drug (Şenel et al. 2000; Aksungur et al. 2004). Sponges provide better mechanical strength than the gels.

Chitosan is a hydrophilic biopolymer obtained by N-deacetylation of chitin, which is the main component of crustacean shells. Recently, much attention has been given to use chitosan in veterinary applications, for its wound healing, tissue regeneration and hemostatic properties and also as a drug delivery system (Klokkevold et al. 1996; Khanal et al. 2000; Özmeriç et al. 2000; Fakhry et al. 2004; Seol et al. 2004; Şenel and McClure, 2004). Alginate derived from brown sea algae is an anionic hydrophilic and biocompatible linear polysaccharide. It has applications as wound dressing, surgical impression materials and bone regenerating agent (Suzuki et al. 1999; Alsberg et al. 2001; Lee and Mooney 2001; Wu et al. 2002; Lin and Yeh 2004). Alginate and some of its composites have been studied for use in a number of biomedical applications for tissue regeneration (Wu et al. 2002; Lin and Yeh 2004).

In this study, polymeric scaffolds in gel and sponge forms were developed for tissue regeneration using chitosan and alginate. Oxytetracycline hydrochloride (OTC), which is a widely used antibiotic in veterinary medicine, and which is similar to tetracycline which has been shown to exert bone regenerating effects, was chosen as the model compound (Park et al. 2000).

2. Investigations and results

2.1. In vitro studies

Chitosan and alginate gels exhibited pseudoplastic flow (Figs. 1a and b). Viscosity was decreased significantly in the presence of OTC with chitosan gels ($p < 0.05$) whereas with alginate gels no change was observed ($p > 0.05$). Cross-linking was found to increase viscosity ($p < 0.05$).

Three-dimensional sponges were obtained with porous structure, layered and reasonably regular network (Fig. 2). The alginate sponge had less and smaller pores and its structure was more dense compared to the chitosan sponges (Fig. 2e).

Significant differences were obtained in water absorption capacities between the sponge formulations (Table 1). The water absorption was decreased in the presence of OTC in chitosan (s-Ch) and alginate (s-Alg) sponges ($p < 0.05$) whereas in chitosan cross-linked (s-Ch/cl) and chitosan/alginate (s-Ch/Alg) sponge formulations it was increased ($p < 0.05$). Water absorption of chitosan sponges was found to be lower than that of alginate sponges ($p < 0.05$) and was decreased with cross-linking ($p < 0.05$). The highest water absorption was obtained with chitosan/alginate sponges.

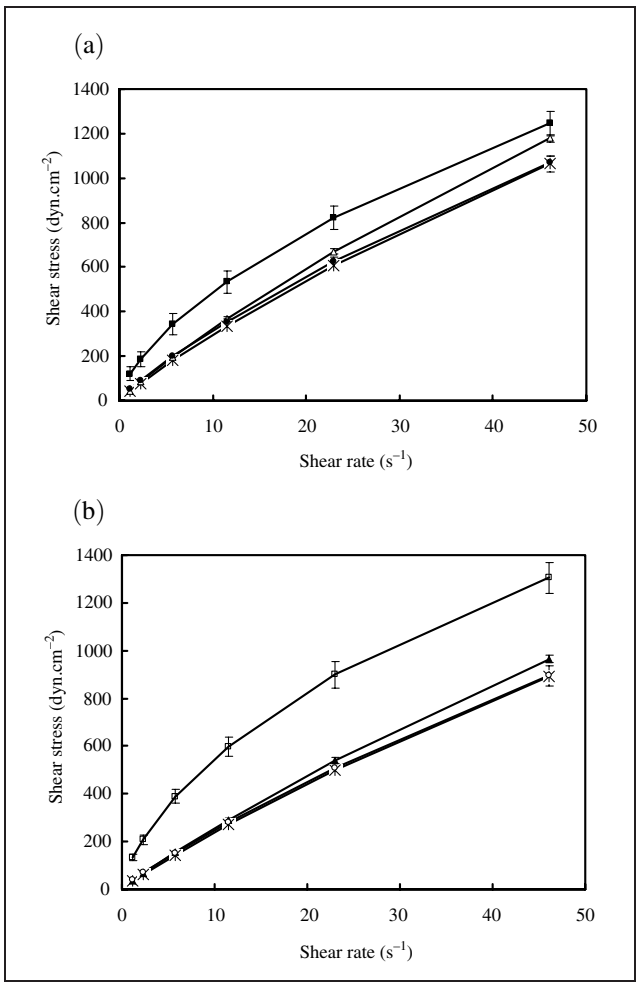


Fig. 1: Flow curves of gel formulations ($n = 3$) in absence (a), and presence (b) of OTC
■: g-Alg, △: g-Ch-aa, ●: g-Ch-dw/cl, ⨯: g-Ch-dw
□: g-Alg-OTC, ▲: g-Ch-OTC-aa, ○: g-Ch-OTC-dw/cl, ⨯: g-Ch-OTC-dw

2.1.1. Release studies

The release profiles of OTC from gel formulations are shown in Fig. 3. The solvent used for preparation of the chitosan gels was found to have a significant effect on release of OTC. The release of OTC from the gels prepared in acetic acid (g-Ch-OTC-aa) ($pH = 2.46$) was higher than that of prepared in distilled water (g-Ch-OTC-dw) ($pH = 3.75$) ($p < 0.05$). The cumulative released percent in 8 h from the formulations, g-Ch-OTC-aa and g-Ch-OTC-dw was 96.0 ± 3.65 and 36.3 ± 3.74, respectively.

Cross-linking did not have a significant effect on release from the chitosan gels ($p > 0.05$). The cumulative released percent from g-Ch-OTC-dw/cl was 31.0 ± 3.48 in 8 h. The release of OTC from the alginate gel (g-Alg-OTC) was found to be lower than from chitosan gel prepared in acetic acid ($p < 0.05$). In 8 h, $36.7 \pm 4.95\%$ was released from g-Alg-OTC.

The release profiles of OTC from sponge formulations are shown in Fig. 4. Chitosan/alginate sponges (s-Ch/Alg-OTC) showed a slower and lower drug release when compared to chitosan, alginate and chitosan/cross-linked sponges ($p < 0.05$). The cumulative released percent in 8 h and 24 h from s-Ch/Alg-OTC was 4.46 ± 0.96 and 10.5 ± 0.87, respectively.

The release from the sponges was found to increase with cross-linking ($p < 0.05$). The released percent in 8 h from chitosan/cross-linked sponges (s-Ch-OTC/cl) and chitosan sponges (s-Ch-OTC) was 15.42 ± 4.03 and 9.96 ± 1.88, respectively. In 24 h, $30.08 \pm 6.32\%$ was released from s-Ch-OTC/cl and $13.29 \pm 12.00\%$ from s-Ch-OTC.

The release of OTC from chitosan sponge (s-Ch-OTC) was found to be similar to that of alginate sponges (s-Alg-OTC) for the first 8 h ($p > 0.05$). The cumulative amount released in 8 h and 24 h from s-Alg-OTC was 9.32 ± 1.24 and $30.9 \pm 6.43\%$, respectively whereas with s-Ch-OTC, it was 9.96 ± 1.88 and $13.29 \pm 12.00\%$ for 8 h and 24 h, respectively.

2.2. In vivo studies

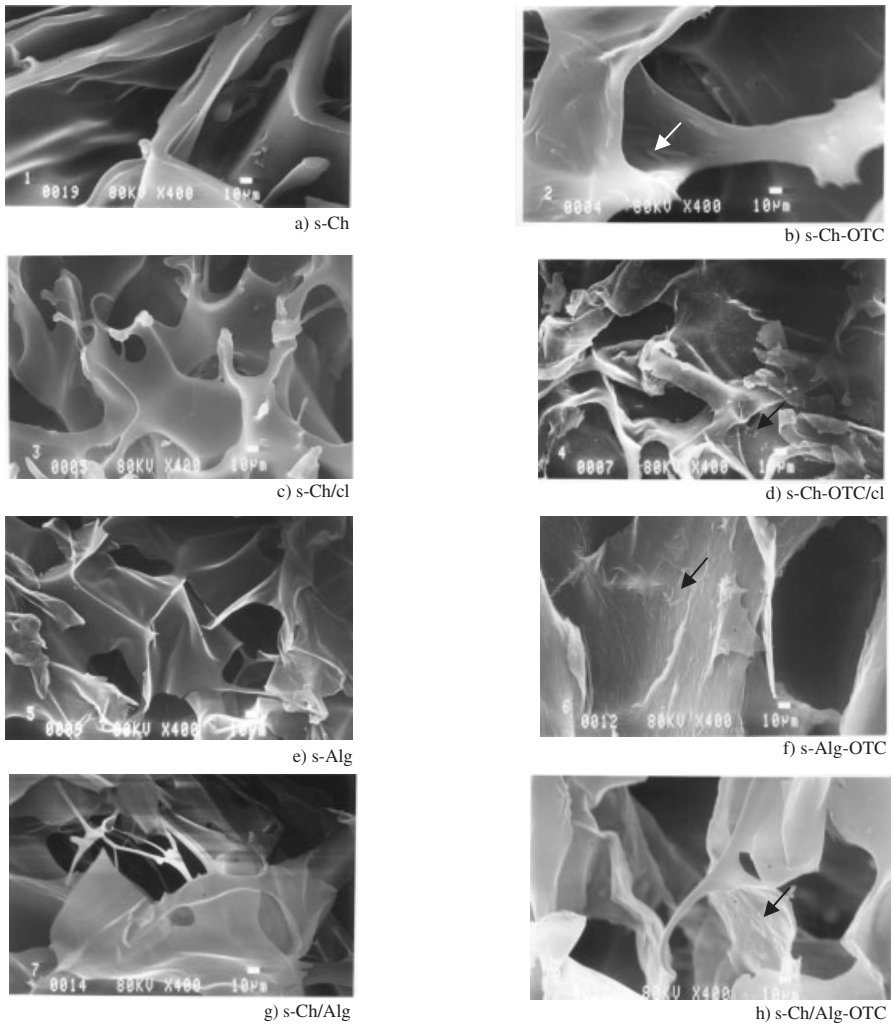
Results of the histological evaluation of the tissue response to the formulations and defect healing are given in Tables 2 and 3. The formulations at the application site were observed to be in close relation with the tissue (Figs. 5 and 6).

A thin fibrous capsule consisting of few layers of spindle shaped fibroblasts was found to surround the scaffolds. Phagocytes were sometimes present adjacent to the scaf-

Table 1: Sponge formulations

Sponge formulation	Cross-linking	Symbol	Polymer concentration of the gel (%)	Water absorption capacity (%)
Chitosan	−	s-Ch*	4	348 ± 24
Chitosan + OTC	−	s-Ch-OTC*	4	204 ± 16
Chitosan	+	s-Ch/cl	4	117 ± 21
Chitosan + OTC	+	s-Ch-OTC/cl	4	191 ± 17
Alginate	−	s-Alg	3	509 ± 45
Alginate + OTC	−	s-Alg-OTC	3	495 ± 33
Chitosan : Alginate (1 : 1)	−	s-Ch/Alg*	3	5073 ± 644
Chitosan : Alginate (1 : 1) + OTC	−	s-Ch/Alg-OTC*	3	7910 ± 153

*Formulations studied *in vivo*



a) s-Ch
b) s-Ch-OTC
c) s-Ch/cl
d) s-Ch-OTC/cl
e) s-Alg
f) s-Alg-OTC
g) s-Ch/Alg
h) s-Ch/Alg-OTC

Fig. 2
Micrographs of sponge formulations (arrows showing the OTC crystals)

folds but this process did not affect new bone formation. No giant cell formation was observed in any experimental group. The cortical site adjacent to the bone plates was totally normal. Remodelling followed reorganization of fibrous tissue and endochondral ossification steps on the application site in all groups. Bone marrow adjacent to the defect area recovered later than the cortical bone itself. Defect site integrity was comperatively better preserved with the sponge than the gel, since degradation was slower in the former. However a slight delay in ossification was closely related with the slow degradation of the sponge in some cases.

On day 7, defect healing process was accelerated in chitosan and alginate scaffolds treated groups when compared to that of control group (Fig. 5). The bottom of the defect area which was in contact with chitosan and/or alginate was partially filled with highly cellular dense connective tissue/fibrous callus immigrating into the scaffold in some locations. Incorporation of OTC into formulations did not show any considerable enhancing effect on defect healing on day 7. However, connective tissue cells of osteogenic potential were forming well organized young bone trabecules in continuity with mature cortical bone with chitosan in one sample (Fig. 5b). The level of organization of the fibrous callus was poorer with the control group when compared to that of with chitosan and/or alginate. Cartilage tissue islands were present in the neighbourhood of cortical bone but no new bone formation was present in the control group.

On day 21, the defect was circumferentially filled with young spongy bone trabecules surrounded by active ostoblasts and resorptive osteoclasts in all groups (Figs. 6b and c). The periosteum was mitotically active and thickened including new bone trabecular or cartilage islands at the periphery of the polymeric matrix holding the implant in place. The thickened connective tissue presented encap-

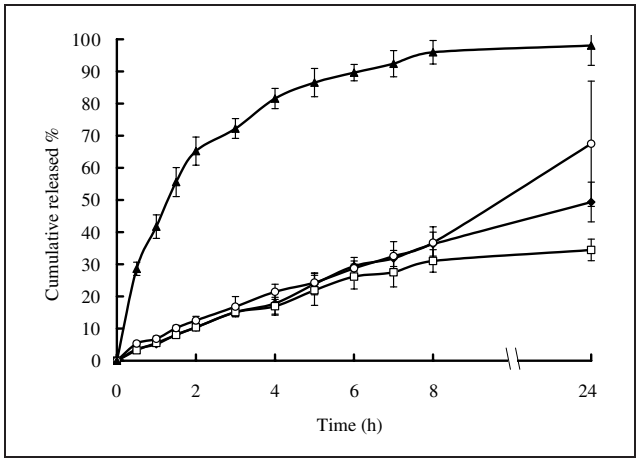


Fig. 3: Release profiles of OTC from gel formulations in distilled water at 37 °C (n = 6);
▲: g-Ch-OTC-aa; □: g-Ch-OTC-dw/cl; ◆: g-Ch-OTC-dw;
○: g-Alg-OTC

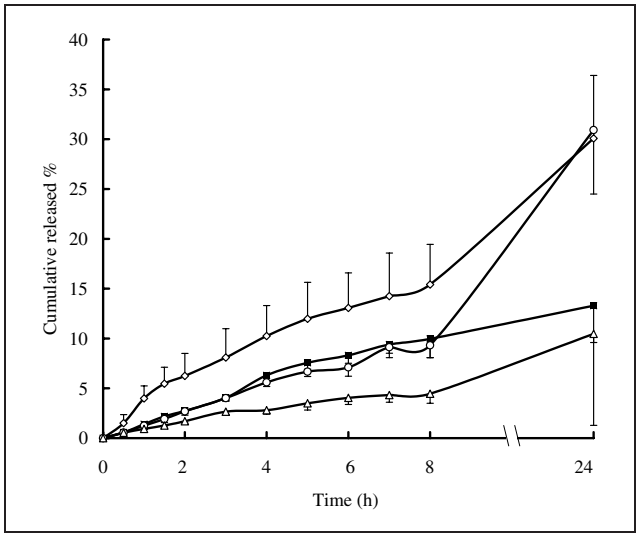


Fig. 4: Release profiles of OTC from sponge formulations in distilled water at 37 °C (n = 7);
■: s-Ch-OTC; ◇: s-Ch-OTC/cl; ○: s-Alg-OTC; △: s-Ch/Alg-OTC

sulation of the polymeric degrading particles in many locations on alginate and/or chitosan groups. The defect healing process was almost on the same level in control and scaffold inserted groups.

On day 60, the defect area was almost ossified; however, a small to medium sized nonossified fibrous tissue was still present in all samples. The size of this nonossified fibrous tissue was more distinctive and large in the control group compared to the scaffold containing groups (Figs. 6d–f). Cortical remodelling was almost completed and spongious remodelling was better with chitosan and alginate (Fig. 6). Dissolving chitosan and alginate particles were observed in close contact with bony trabecules.

Bone marrow recovery initiated by the replacement of fibrous connective tissue that was than restored by original marrow elements starting from 21 days of application. On day 60, bone marrow remodelling followed cortical remodelling and stromal elements appeared in chitosan and alginate scaffolds treated groups (Figs. 6d–f).

3. Discussion

3.1. In vitro studies

3.1.1. Development of formulations

Gels were prepared at two different pH values (for g-Ch-dw: 4.80 ± 0.04; g-Ch-aa: 2.95 ± 0.07) using soluble chitosan. The chitosan gel formulations were prepared at 4% (w/v) concentration which was reported to be suitable for application (Aksungur et al. 2004).

Table 2: Results of the histological evaluation of the tissue response to gel formulations and defect healing* (n = 4)

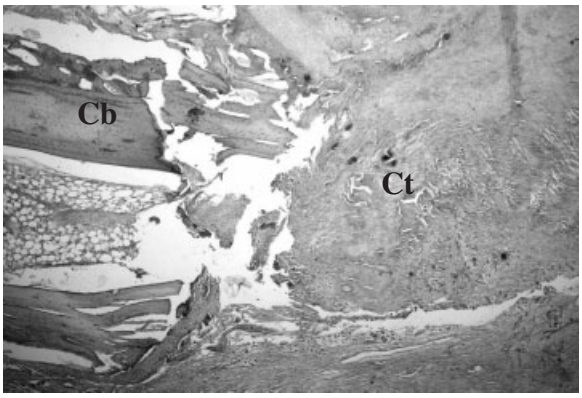
	Parameters	Control			g-Ch-OTC-dw			g-Ch-dw			g-Alg-OTC			g-Alg		
		Day 7	Day 21	Day 60	Day 7	Day 21	Day 60	Day 7	Day 21	Day 60	Day 7	Day 21	Day 60	Day 7	Day 21	Day 60
Category 1 Bone defect repair	New bone formation in the defect	0, 0, 0, 0	1, 2, 2, 1	2, 2, 2, 2	1, 0, 1, 0	1, 2, 1, 1	2, 2, 2, 1	1, 0, 1, 1	2, 2, 2, 2	2, 2, 2, 2	1, 0, 1, 1	2, 2, 2, 1	3, 2, 2, 2	1, 0, 1, 1	2, 2, 2, 1	2, 2, 2, 2
	Cortex remodelling	0, 0, 0, 0	1, 1, 1, 0	3, 2, 2, 2	0, 0 0, 0	1, 1, 1, 0	3, 3, 2, 3	1, 0, 0, 1	1, 1, 1, 1	3, 2, 2, 3	0, 0, 1, 0	2, 1, 1, 2	3, 3, 3, 2	1, 0, 0, 1	1, 1, 1, 1	3, 2, 2, 3
Category 2 Tissue response	Fibrous connective tissue formation	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	0, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 2, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 0, 1	1, 1, 1, 1	0, 0, 0, 0
	Inflammatory cellular infiltration	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	1, 1 1, 1	1, 1, 1, 1	0, 0, 0, 0	0, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1,1, 1, 1	1, 1, 1, 0	0, 0, 0, 0

* Tissue response to the formulations and defect healing were scored according to Table 3

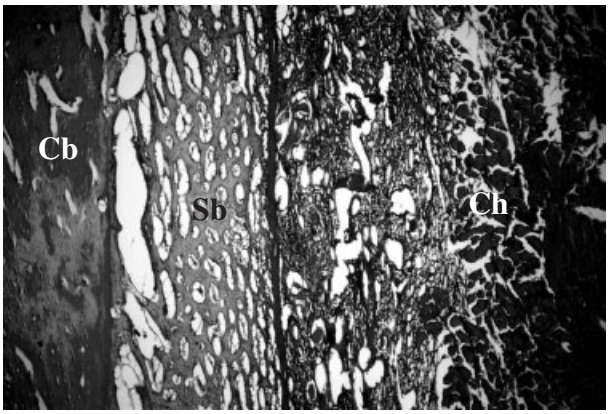
Table 3: Results of the histological evaluation of the tissue response to sponge formulations and defect healing* (n = 4)

	Parameters	Control			s-Ch/Alg-OTC			s-Ch/Alg			s-Ch-OTC			s-Ch		
		Day 7	Day 21	Day 60	Day 7	Day 21	Day 60	Day 7	Day 21	Day 60	Day 7	Day 21	Day 60	Day 7	Day 21	Day 60
Category 1 Bone defect repair	New bone formation in the defect	0, 0, 0, 0	1, 2, 2, 1	2, 2, 2, 2	1, 0, 1, 0	2, 2, 1, 2	2, 2, 2, 2	1, 0, 1, 1	2, 1, 1, 2	2, 2, 2, 2	1, 0, 1, 0	1, 2, 2, 1	1, 2, 2, 1	1, 0, 1, 0	2, 2, 2, 2	2, 1, 2, 1
	Cortex remodelling	0, 0, 0, 0	1, 1, 1, 0	3, 2, 3, 2	0, 1, 1, 0	2, 2, 2, 2	3, 2, 3, 2	1, 0, 0, 1	2, 2, 2, 1	3, 2, 3, 2	0, 0, 0, 0	1, 1, 1, 1	2, 3, 3, 2	1, 0, 0, 1	1, 1, 1, 1	3, 2, 3, 2
Category 2 Tissue response	Fibrous connective tissue formation	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	1, 0, 1, 0	1, 1, 1, 1	1, 1, 1, 1	0, 1, 1, 0
	Inflammatory cellular infiltration	0, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0	1, 0, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0	1, 1, 1, 1	1, 1, 1, 1	0, 0, 0, 0

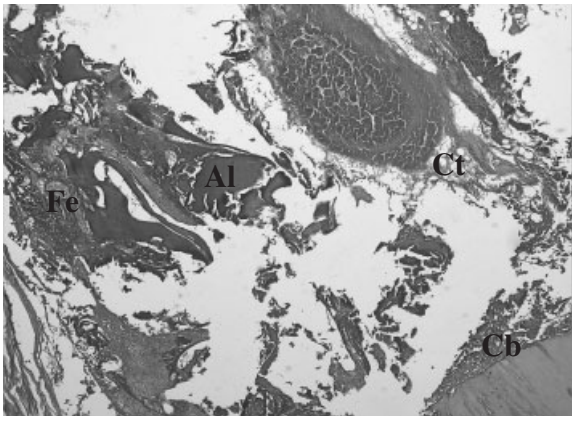
* Tissue response to the formulations and defect healing were scored according to Table 3



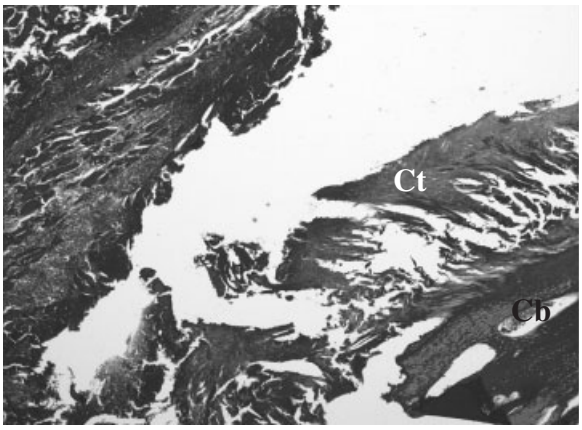
a) Control (40x, H&E)



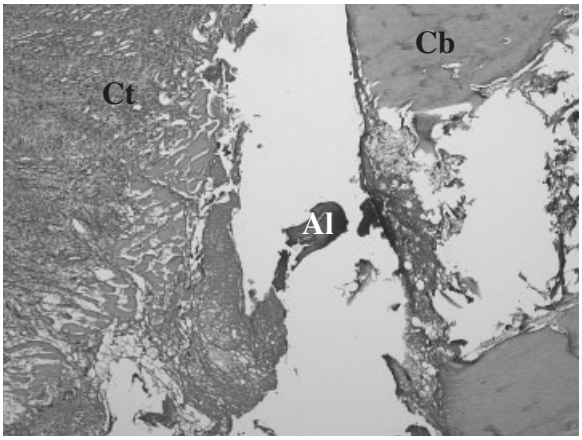
b) g-Ch-dw (40x, MT)



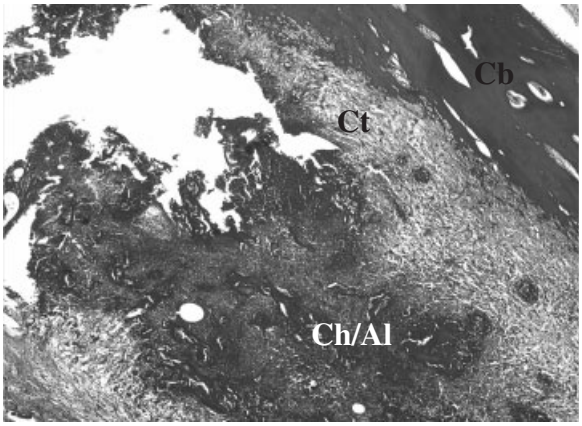
c) g-Alg (40x, H&E)



d) s-CH-OTC (40x, MT)



e) g-Alg-OTC (40x, H&E)



f) s-Ch/Alg-OTC (40x, MT)

Fig. 5: Histological appearance of bone specimens on Day 7. Mature bone is stained in red, new bone in yellow and the collagen fibers of connective tissue in blue with Masson's trichrome. Note the thin fibrous encapsulation and close contact of dissolving chitosan and alginate particles with bony trabecules, Cb: Compact bone (in red), Sb: Spongy bone, Ct: Connective tissue, Ch: Chitosan, Al: Alginate, Ch/Al: Chitosan/Alginate, Fe: Fibrous encapsulation, MT: Masson's Trichrom, H & E: Haematoxylin & Eosin

Alginate gels at different concentrations (1 to 4% w/v) were prepared and 3% (w/v) concentration was found to have appropriate rheological properties from the applicability point of view. Cross-linking was not applied to alginate gels due to incompatibility of OTC with the cross-linking agents (e.g. magnesium and calcium) which are commonly used for alginate.

An ideal tissue engineering scaffold is expected to act as a template for tissue growth in three dimensions by having an interconnected macroporous network for tissue ingrowth, vascularization and nutrient delivery (Vats et al. 2003). The sponge formulations prepared in our study appeared to be were highly porous and had interconnected pore structure (Fig. 2). The water absorption capacity of the formulations was found to be related to the porous structure of the sponges. The alginate sponges showing more dense porous structure exerted higher water absorption when compared to that of chitosan sponges (Fig. 2). Gelation was observed within 30 min after exposure to water. Combination of two polymers resulted in higher water absorption capacity and

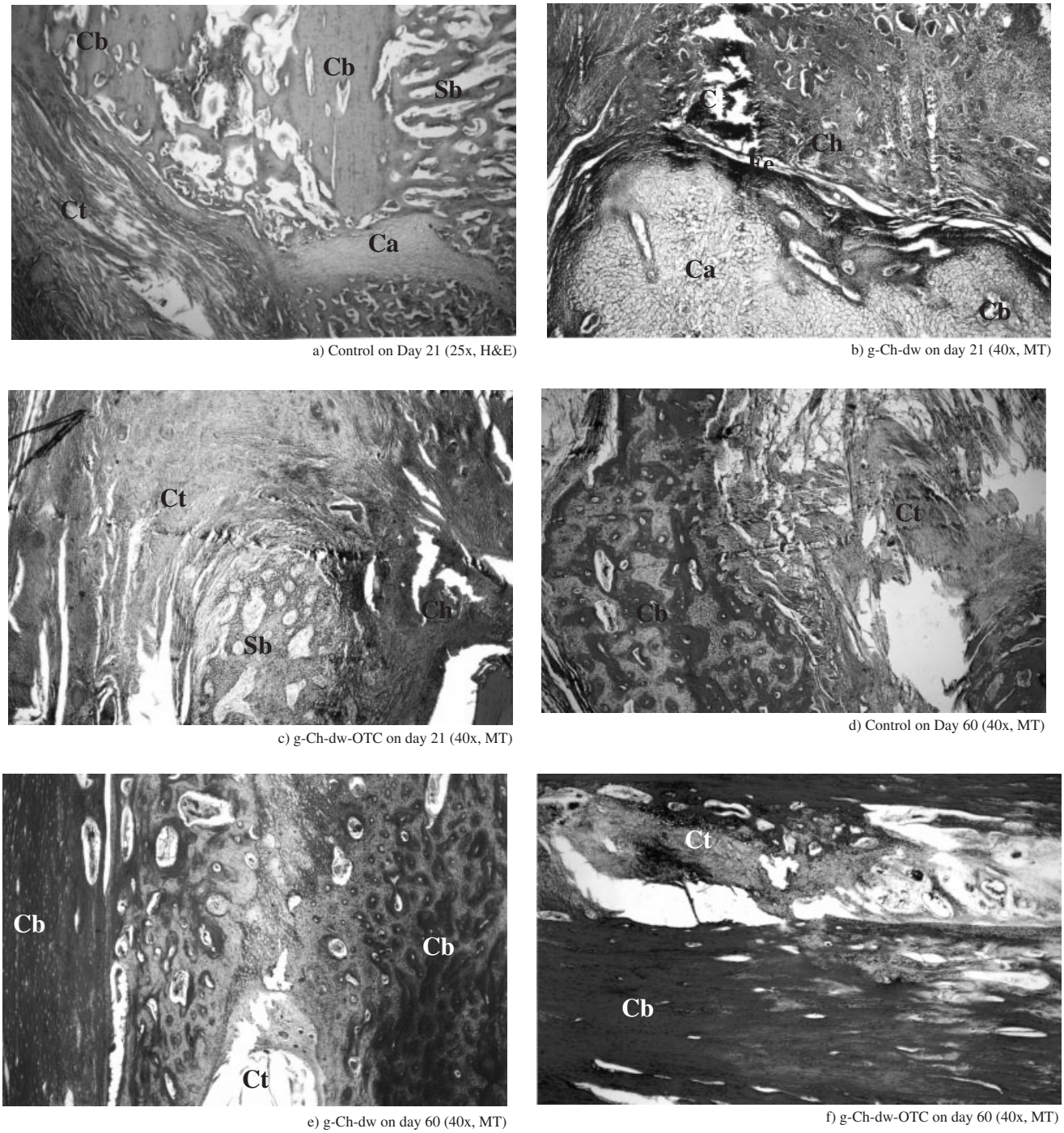


Fig. 6: Histological appearance of bone specimens on Days 21 and 60. Mature bone is stained in red, new bone in yellow and the collagen fibers of connective tissue in blue with Masson's trichrome. Fibrous tissue is gradually replaced by cartilage tissue and bone from day 21 to 60; however, a nonossified fibrous tissue is still present in all samples. The size of this fibrous tissue is smaller (d–f), cortical remodelling is almost completed and spongious remodelling is better with biomaterials comparing to control (a–f), Cb: Compact bone, Sb: Spongy bone, Ct: Connective tissue, Ca: Cartilage, Ch: Chitosan, Fe: Fibrous encapsulation, MT: Masson's Trichrom, H & E: Haematoxylin & Eosin

sponge formulations remained intact for longer than 24 h. Similar results were reported by Li et al. (2005) who prepared chitosan-alginate hybrid scaffolds for bone tissue engineering. The structural stability observed for the chitosan-alginate sponges can be explained by the interaction of amine groups on chitosan with carboxyl groups on alginate which prevents the protonation of amino groups on chitosan. Furthermore, it is likely that the carboxyl groups on alginate buffer the solution and slow down chitosan degradation.

3.1.2. Release studies

The solvent used for the preparation of chitosan gels was found to have a significant effect on release profiles of OTC. The release of OTC from the gels prepared in acetic acid was higher than that of prepared in distilled water ($p < 0.05$). In 2 h, the release of OTC was $65.21 \pm 4.38\%$ from the chitosan gel prepared in acetic acid ($pH = 2.5 \pm 0.1$) whereas $10.36 \pm 0.99\%$ was released from the gel prepared in distilled water ($pH = 3.8 \pm 0.1$). This can be explained by the incresed charged density due to the protonation of the free

amino groups of chitosan at lower pH values. This protonation leads to chain repulsion, diffusion of water into the gel matrix and swelling. Thus, the release of the drug from the gel increases (Khalid et al. 2002). Similar results were obtained with pH-sensitive chitosan-polyvinyl pyrrolidone hydrogels prepared for antibiotic delivery (Risbud et al. 2000). It was shown that the swelling degree and porosity of the hydrogels increased under acidic conditions and drug release increased due to diffusion of release medium into the gel matrix.

The release of OTC from alginate gels was found to be lower than that of chitosan gel prepared in acetic acid ($p < 0.05$). This can be explained by the differences in ionic charge of the polymer used as well as the difference in viscosity of the formulations. An ionic interaction between the positively charged OTC and negatively charged alginate can result in a decrease in release. Higher viscosity of alginate gel can slow down the penetration of water into gel which would also decrease the release of the drug.

In the first 8 h, no significant difference between the chitosan and alginate sponges ($p > 0.05$) was observed. Cross-linking was found to increase the release from the chitosan sponges. There are studies showing that the release of drug was decreased by cross-linking but also other studies reporting no difference after cross-linking (Lee et al. 2000; Leffler and Müller 2000). The increase in release observed in our study can be attributed to the preparation method. For cross-linking, the TPP solution is added on the sponge incorporated with OTC and then the sponge is freeze-dried again. This process might affect the dissolution of OTC in the sponge which would result in an increase in release.

Chitosan/alginate sponges showed a slower and lower drug release when compared to chitosan, alginate and chitosan/cross-linked sponges ($p < 0.05$). This can be due to the ionic interaction between chitosan and alginate.

3.2. In vivo studies

Polymeric biomaterials that are implanted into the bone launch a series of tissue reactions depending on many factors including their degradation properties. The first category of tissue response is initiated by the surgical procedure itself and the second one by the biomaterial. A fibrous capsule of variable thickness surrounds the polymer regardless of the polymer type as long as the scaffold is nondegradable (Anderson et al. 1992). In this study the scaffolds at the application site were observed to be in close relation with the tissue surrounding by a thin layer of fibrous capsule during their degradation process. Neither fibrosis nor foreign body reaction was observed in any of the experimental groups. Both alginate and chitosan were highly biocompatible. The locations of the scaffolds were sometimes observed as voids in the defect area. Defect site integrity was comperatively better preserved with the sponge then the gel, since degradation was slower in the former. However a slight delay in ossification was closely related with the slow degradation of the sponge.

No neutrofil migration was observed in any of the alginate and chitosan treated groups, indicating the biocompatibility of the biomaterials used in our study. Alginate and chitosan scaffolds provided good guidance for ossification with no superiority to each other. On day 7, the defect healing process was accelerated in chitosan and alginate scaffolds treated groups when compared to that of the control group; however the healing process was almost on the same level in control and scaffold treated groups on day 21. The osteoconductive promoting effect may be important in the initial phase of ossification. On the other hand, dissolving chitosan and alginate particles were observed in close contact with bony trabecules and bone marrow remodelling followed cortical remodelling and stromal elements appeared in the scaffold treated groups, but not in control group on day 60. These observations confirm the presence of a healthy degradation-ossification and remodelling process due to both alginate and chitosan on day 60.

As a conclusion, chitosan and alginate gel and sponges can be suggested as promising delivery systems for tissue regeneration in veterinary medicine which provide a biocompatible and biodegradable scaffold for bone cells to grow in and due to their bioadhesive properties extending the residence time at the application site. These systems can be incorporated with a drug, and they release the drug in an extended fashion.

4. Experimental

4.1. Materials

Chitosan (Protasan UP CL-213, deacetylation degree: 86%; Mol.Wt.: 272,000 Da; FMC-Biopolymer, Norway), sodium alginate (medium viscosity, Sigma, USA) and glacial acetic acid (E. Merck, Germany) were used as recieved. Tripolyphosphate pentasodium salt (TPP; Sigma, USA) was used for cross-linking. OTC was kindly provided by Pfizer İlaçları, Ltd. Şti., Turkey.

4.2. In vitro studies

4.2.1. Preparation of formulations

Gel formulations are given in Table 4. Chitosan gel formulations at 4% (w/v) concentration were prepared either in distilled water or in dilute acetic acid (1% v/v). For cross-linking, 0.05% (w/v) TPP was dissolved in distilled water before the addition of polymer. Alginate gel formulations were prepared at 3% (w/v) concentration in 1% (v/v) acetic acid. 0.5% (w/w) OTC was incorporated into gels.

Sponge formulations given in Table 1 were prepared by a freeze-drying process. 4% (w/v) chitosan gel, 3% (w/v) alginate gel and 3% (w/v) chitosan : alginate (1 : 1) gel formulations were prepared in distilled water. 0.5% (w/w) OTC was incorporated into the gels. The gels were frozen at $-22\,^{\circ}C$ for 24 h and then lyophilized in a freeze dryer (Labconco freeze-drier system-Freezon 4.5) for 24 h. Chitosan sponges were crosslinked with 5% (w/v) TPP and were freeze-dried again. Sponge formulations without OTC were also prepared.

4.2.2. Viscosity of the gels

Viscosity measurements of the gels were performed on a Brookfield digital viscometer (Model DV-II, cone-SD1) at room temperature.

4.2.3. Morphology of sponges

The sponge structures were examined under a scanning electron microscope (Jeol SEM-ASID 10, Japan) at an acceleration voltage of 80 KV. The specimens were fixed on metal stubs with a double-sided adhesive band and sputtered with a 100 Angstrom thick layer of gold in a BIO-RAD sputter apparatus.

4.2.4. Water absoption capacity of sponges

Sponge samples (0.05 g) were accurately weighed and transferred into a vial containing 20 mL distilled water and shaken at 37 °C in a horizontal shaker until the maximum weight was reached (~10 min, determined from preliminary experiments), then the sample was weighed after carefully removing excess water. The water absorption capacity was calculated using Eq. (1):

$$\text{Water absorption capacity } (\%) = \frac{W_1 - W_0}{W_0} \times 100 \qquad (1)$$

where, W_0 is the initial weight, and W_1 is the maximum weight.

4.2.5. In vitro drug release from gels and sponges

Release from gels and sponges were studied using Franz diffusion cells with a 2.52 cm^2 diffusion area and 20 mL receptor (distilled water) volume. Samples of 1 mL were taken from the medium at certain time intervals and replaced with the same amount of distilled water. The samples were assayed spectrophotometrically at 276 nm, using a UV 160A Shimadzu Spectrophotometer (Japan). One-way analysis of variance (ANOVA) was performed for statistical analysis.

Table 4: Gel formulations

Formulation	Cross-linking	Symbol	Polymer conc. (%)	Medium	pH
Chitosan	−	g-Ch-dw*	4	Distilled water	4.80 ± 0.04
Chitosan + OTC	−	g-Ch-OTC-dw*	4	Distilled water	3.75 ± 0.07
Chitosan	+	g-Ch-dw/cl	4	Distilled water	4.82 ± 0.05
Chitosan + OTC	+	g-Ch-OTC-dw/cl	4	Distilled water	4.13 ± 0.06
Chitosan	−	g-Ch-aa	4	1% acetic acid	2.95 ± 0.07
Chitosan + OTC	−	g-Ch-OTC-aa	4	1% acetic acid	2.46 ± 0.07
Alginate	−	g-Alg*	3	1% acetic acid	4.20 ± 0.10
Alginate + OTC	−	g-Alg-OTC*	3	1% acetic acid	4.09 ± 0.09

*Formulations studied *in vivo*

Table 5: Histological scoring system (Keskin et al. 2005)

Scores	Category 1 Bone defect repair			
	New bone formation in the defect	Graft resorption	Marrow changes	Cortex remodelling
3	Full bone formation in the defect	Full resorption	Adult type fatty marrow	Full remodeling cortex
2	Moderate bone formation (>50%)	Moderate resorption (>50%)	>50% replaced by new tissue	Moderate remodeling (>50%)
1	Mild bone formation (<50%)	Mild resorption (<50%)	<50% replaced by new tissue	Mild remodeling (<50%)
0	No new bone	No resorption	Fibrous tissue or red	No remodelling

	Category 2 Tissue response	
	Fibrous connective tissue formation	Inflammatory cellular infiltration
4	Severe deposition of dense collagenous connective tissue around implant	Severe cellular infiltrate response to implant or tissue necrosis at or around the site
3	Disruption of normal tissue architecture and presence of moderately dense fibrous connective tissue	Presence of large numbers of lymphocytes, macrophages and foreign body giant cells, also notable presence of eosinophils and neutrophils
2	Presence of moderate connective tissue	Presence of several lymphocytes, macrophages with a few foreign body giant cells and a small foci of neutrophils
1	Presence of delicate spindle shaped cells or mild fibroplasia	Presence of a few lymphocytes or macrophages, no presence of foreign body giant cells, eosinophil or neutrophils
0	No difference from normal control tissue, no presence of connective tissue at or around implant site	No difference from normal control tissue, no presence of macrophages, foreign body cells, lymphocytes, eosinophils or neutrophils at or around implant site

4.3. In vivo studies

In vivo studies were performed on White New Zealand rabbits. All protocols were approved by the Ankara University Faculty of Veterinary Medicine Animal Ethics Committee (#2002/03). After anesthetizing the animals with intramuscular injection of 0.1 mL/kg xylazine hydrochloride (23.32 mg/mL, Bayer Turk Kimya San. Ltd. Sti., Turkey) and 10 mg/kg ketamine hydrochloride (100 mg/mL, Ketamidor, Richter Pharma, Austria), bilateral antebrachium (forearm) of rabbits were shaved and disinfected with Salvex (15% Savlon ve 1.5% chlorhexidine gluconate) (Drogsan, Turkey). 1 cm standard bicortical bone defects were surgically prepared with osteotomy. Formulations indicated with asterisk in Tables 1 and 4 were applied on the surgically formed bone defects of radius. Connecting tissue under the skin was closed by using 4/0 polyglactine 910 (Vicryl, Ethicon, UK), and the skin was closed with 3/0 polyester (Ethibond, Ethicon, UK). The untreated defects were taken as the control group.

Rabbits were sacrificed on days 7, 21 and 60, and the effect of the formulations on healing over time was examined histologically. Four rabbits per experimental day were used for each formulation. Bone specimens were retrieved, fixed in 10% phosphate buffered formalin (pH 7.0) at room temperature, rinsed in buffer, and decalcified in De Castro solution. They were then dehydrated in a graded series of ethanol before embedded in paraffin. Sections five to seven micrometer thick were prepared with a rotary microtome (Microm, HM 360, Germany). Haematoxylin & Eosin, and Goldners Masson Trichrome stained sections were evaluated for overall morphology, new bone formation and tissue response. Stained sections (a minimum of ten sections obtained from different levels of each tissue) were examined by at least two investigators and the specimens were documented under a Leica DMR light microscope (Germany). The images were taken via a Leica DC500 digital camera. Tissue response to the formulations and defect healing were separately scored (Table 5) (Keskin et al. 2005). The maximum expected score is 6 for bone defect repair (3 for new bone formation in the defect and 3 for cortex remodelling) and 0 for tissue response (0 for fibrous connective tissue formation and 0 for inflammatory cellular infiltration).

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