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Research Article

Histopathological evaluation of the effect of microspheres with different natural bioactive components (*Ganoderma lucidum* and *Inula graveolens*) on osteoblastic activity in rats with experimental bone wounds

Ali Batuhan Bayırlı[1*], Serhat Sezgin[2], Ezgi Eren Belgin[3], Leyla Tekin[4], Hüseyin Çiçek[3], Ayşegül Demirbaş[5], Cankız Gizem Delibalta[3], Fatma Yılmaz[6]

[1]Muğla Sıtkı Koçman University, Faculty of Dentistry, Department of Periodontology, Muğla
[2]Muğla Sıtkı Koçman University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Muğla
[3]Muğla Sıtkı Koçman University, Faculty of Science, Department of Chemistry, Muğla
[4]Muğla Sıtkı Koçman University, Faculty of Medicine, Department of Pathology, Muğla
[5]Ege University, Faculty of Dentistry, Department of Restorative Dentistry, İzmir
[6]Muğla Sıtkı Koçman University, Faculty of Dentistry, Department of Restorative Dentistry, Muğla

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Abstract: This study aimed to analyse the effects of microspheres containing *Ganoderma lucidum* and *Inula graveolens* on bone healing in the alveolar socket after tooth extraction in Wistar rats. In this study, chitosan-coated and uncoated hydrogel microspheres were produced using sodium alginate-gelatine by dispersed phase gelling and crosslinking method in microsphere (MS) production. *G. lucidum* (GL) and *I. graveolens* (IG) extracts were entrapped in these microspheres. 126 healthy female rats were randomly divided into 7 different groups. The groups were named according to the microsphere placed in the alveolar socket after extraction. The effects of these microspheres on the healing of the alveolar bone in the groups were evaluated on the 7th day, the 14th day and the 28th day. Immunohistochemical analysis was used to assess bone healing. A statistically significant difference was observed between the negative control group and MS + Chitosan + IG group and between the MS group and MS + Chitosan + IG group in terms of bone formation percentages on the 28th day ($p<0.05$). The results showed that *G. lucidum* and *I. graveolens* in combination with chitosan can enhance the bone healing of the alveolar socket after extraction.

1. INTRODUCTION

Bone tissue, the main structure of the skeletal system, is a specialised type of connective tissue with a calcified extracellular matrix containing characteristic cells (Gartner *et al.*, 2011). This tissue, which consists of structural matrix proteins including type I collagen, bone sialoprotein, osteocalcin, osteonectin, osteopontin, proteoglycans, growth factors and serum proteins, is a dynamic structure with regeneration capacity in which formation and resorption occurs (Lindhe *et al.*, 2009; Gartner *et al.*, 2011; Hollý *et al.*, 2021). There are four types of cells in bone which include osteoblasts, osteocytes, osteoclasts and osteoprogenitor cells (Florencio-Silva *et al.*, 2015). Osteoblasts secrete proteins including type I collagen, osteocalcin, osteonectin and

*CONTACT: Ali Batuhan BAYIRLI batubayirli48@gmail.com Muğla Sıtkı Koçman University, Faculty of Dentistry, Department of Periodontology, Muğla, Türkiye

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signalling proteins including platelet-derived growth factor (PDGF), bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β) which play an important role in bone regeneration (Yoshiko *et al.,* 2007; Kim *et al*., 2020). These proteins play a role in bone regeneration that may occur in the event of alveolar bone injury (Davison *et al*., 2016; Majidinia *et al*., 2018).

In the alveolar socket, which undergoes a series of tissue healing stages after tooth extraction, a gateway is initially formed that facilitates the spread of bacteria. As a result of this threat, a defense line is formed by immune cells. Due to the surgical procedure, it starts to fill with blood containing cells from the severed vessels (Cohen & Cohen-Lévy, 2014). These cells together with platelets form a blood clot covering the socket in the first 24 hours. The inflammatory reaction stimulates the aggregation of cells to form granulation tissue. In the first 72 hours after extraction, the clot starts to disintegrate as granulation tissue starts to leak into the clot from the base and periphery of the extraction socket (Darby *et al*., 2009). An immature and provisional connective tissue matrix is then formed in the socket. After this stage, woven bone and lamellar bone formation occurs (Irinakis, 2006; Shah *et al*., 2019; Min *et al*., 2020; Miranda *et al*., 2020). Bone wound healing process by creating an experimental wound area after tooth extraction has been analysed in previous studies (Okamoto & Russo, 1973; Carvalho *et al*., 1997). This healing process is seen in three histological stages: the exudative stage with clot formation, the proliferative stage with connective tissue formation and the reparative stage including ossification (Rodrigues *et al*., 2016; Hassumi *et al*., 2018). The healing process of the alveolar bone wound area in rats is completed in 28 days (Carvalho *et al*., 1997).

Different materials and treatment methods such as bone grafts (autogenous, allogenic, xenogeneic, and alloplastic bone grafts), demineralised dentin matrix, synthetic materials, platelet-rich plasma application and plant extract therapy are used to increase the regeneration of bone tissue in bone defects and formed wound areas (Troiano *et al*., 2018; Santos *et al*., 2019; Grawish *et al*., 2022; Karayürek *et al*., 2019; Shi *et al*., 2022; Wang *et al*., 2021). *Ganoderma lucidum* Karst (1881) (Ganodermataceae) and *Inula graveolens* (L.) Desf. (Asteraceae) are among the fungi and plants that can be used for regeneration of bone wound site. *G. lucidum* is a fungus species that contains glycoproteins, polysaccharides, triterpenoids, steroids, alkaloids, benzopyran derivatives, benzoic acid derivatives and some minerals such as potassium, calcium, phosphorus, magnesium, selenium, iron, and zinc (Benzie & Wachtel-Galor, 2011; Baby *et al*., 2015; Sohretoglu & Huang, 2018). Due to its anti-inflammatory, antimicrobial, antioxidant and immunostimulant effects, *G. lucidum* has been used in the treatment of diseases such as cancer, immune system disorders, neurodegenerative diseases, hepatitis, hypertension, chronic bronchitis, bronchial asthma (Ma *et al*., 2015; Cör *et al*., 2018; Laçin *et al*., 2019). Studies in rats have reported that *G. lucidum* prevents the loss of bone density and is effective in the treatment of bone defects (Miyamoto *et al.*, 2009; Laçin *et al*., 2019). *I. graveolens* is an erect plant from the Asteraceae family with a sticky texture and a strong odour (Mitic *et al*., 2016). Essential oil and extracts of *I. graveolens* contain polyphenol, tannin, flavonoid, oil, steroidal triterpenoids, sesquiterpene and anthraquinone. These have been reported to give *I. graveolens* antimicrobial, antioxidant, antiproliferative, antipyretic, analgesic, and anti-inflammatory activity (Sevindik & Paksoy, 2017; Al-Snafi, 2018; Koc *et al*., 2021).

Controlled drug release systems are systems that can maintain the active substance regionally or systematically at a predetermined therapeutic rate in the target area for an appropriate period (Tüylek, 2019). Microspheres are now widely used in controlled drug delivery systems (Tüylek, 2017). Microspheres are monolithic microcarriers that carry the active substance in the form of particles at molecular level, have different physicochemical properties and have a diameter size ranging from a few microns to millimetres (Singh *et al*., 2013; Singh *et al.,* 2014). Polysaccharides and natural polymers such as gelatine, chitosan and alginate can be used for production of microspheres. These materials are preferred due to their biocompatibility, biodegradability, and high loading efficiency (Menon *et al*., 2014; Alsmadi et al., 2020). Studies

have shown that mixtures of chitosan-alginate-gelatine polymers give favourable results in controlled release drug systems (Kuo & Wang, 2013; Jia *et al*., 2015; Afzal *et al*., 2018). Chitosan has also been reported to contribute to bone regeneration in drug delivery systems (Kim *et al.*, 2016; Yadav *et al*., 2021).

In this study, chitosan-coated and uncoated polymers were prepared using sodium alginate and gelatine by the dispersed phase gelling and cross-linking method in microsphere (MS) production, and pharmaceutically active extracts of *G. lucidum* and *I. graveolens* were entrapped in these microspheres. The effects of these microspheres on alveolar bone healing after tooth extraction in rats were evaluated on days 7, 14 and 28. Bone wound healing was evaluated by objective criteria using histopathological and immunohistochemical analysis. The study was designed to evaluate the effect of the bioactive components used in regenerating alveolar bone following extraction.

2. MATERIAL and METHODS

2. 1. Preparation of the Bioactive Agent Extract

Dried *G. lucidum* mushrooms were first cut into small pieces and then reduced in size using a laboratory type shredder. The crushed mushrooms were placed in a flask containing ethanol and the first stage extraction was carried out for 24 hours at room temperature in an incubator shaker (24^{o}C, 125 rpm). The mixture was then filtered, and ethanol solution was obtained. The same extraction process was repeated twice more with the solid part (fungus). The ethanol solution containing the extract obtained from the different extraction steps were then combined. The resulting total solution was placed in a rotary evaporator and the ethanol was removed for 72 hours at 40^{o}C. The extract was then transferred to a beaker and subjected to lyophilisation for 48 h. Extract was stored at +4°C until use. The same procedures were followed for *I. graveolens*.

2.2. Preparation of Microspheres

3% solution of sodium alginate (Sigma Aldrich, medium viscosity) and 1% solution of gelatine (Huaxuan, 80-120 bloom) were prepared, mixed and homogenised. For the preparation of loaded spheres, *I. graveolens* and *G. lucidum* extracts were added to the homogenised polymer solution at a rate of 1:20 (w/v). Extracts were homogeneously distributed in the solution by using an ultrasonic sonicator. For the preparation of the unloaded spheres, this step was omitted. After adjusting the pH of the mixture to 7.0, mixture was drawn into the syringe pump injector and dropped into the 0.15 M $CaCl_2$ solution at a speed of 0.1 mL and height of 10 cm under stirring to form microspheres. To produce spheres coated with chitosan, 5 ml of the chitosan solution was added to the $CaCl_2$ solution in this step. This step has been skipped in the case of non-chitosan coated sphere production. Stirring was continued for a further 3 minutes. The spheres were then filtered and the $CaCl_2$ solution removed. They were then washed three times with distilled water, transferred to Falcom tubes and lyophilised at -80°C for 48 hours (Belgin *et al*., 2022)

Following the above protocol, a total of six types of microspheres were prepared: non-chitosan coated microspheres (MS), chitosan coated microspheres (MS + Chitosan), non-chitosan coated microspheres containing *G. lucidum* (MS + GL), chitosan coated microspheres containing *G. lucidum* (MS + Chitosan + GL), non-chitosan coated microspheres containing *I. graveolens* (MS + IG) and chitosan coated microspheres containing *I. graveolens* (MS +Chitosan + IG).

2.3. Experimental Animal Study

2.3.1. *Rat model*

Ethical approval for this study was obtained from Muğla Sıtkı Koçman University Animal Experimentation and Research Centre, Local Ethics Committee for Animal Experimentation (MUDEM-HAYDEK), decision number 19/21. The manuscript was prepared according to ARRIVE guidelines. In the power analysis of the study, at a 95% confidence level (α=0.05),

with 95% power, the minimum sample size was obtained as N=126 for 21 groups and n=6 observations. In this study, 126 female Wistar rats aged 2-3 months were used. The rats were obtained from the Animal Experimentation and Research Centre of Muğla Sıtkı Koçman University. The experiments were performed at this centre. During the study period, all animals were allowed to acclimate to their environment one week before the start of the study. All rats were housed in plastic cages with adequate food and water, a temperature of 19-23°C, humidity of 30-70%, and a 12-hour day/night cycle.

2.3.2. *Designing the experimental model*

3 mg/kg of Xylazine and 90 mg/kg of Ketamine HCl, was used intraperitoneally to anaesthetise the rats. Experimental wounds were created in the alveolar bone of all rats by extraction of the left maxillary incisor. The rats in the study groups received microspheres containing bioactive agents. The microspheres were placed in the alveolar socket in such a way that it was filled and closed. Control and negative control groups were formed. The control groups received chitosan-coated and non-chitosan-coated microspheres without bioactive agents, while the negative control groups received no microspheres (see Table 1).

Table 1. Study groups.

Day 7 Groups	Day 14 Groups	Day 28 Groups
Negative Control (n=6)	Negative Control (n=6)	Negative Control (n=6)
MS+Chitosan (control) (n=6)	MS+Chitosan (control) (n=6)	MS+Chitosan (control) (n=6)
MS (control) (n=6)	MS (control) (n=6)	MS (control) (n=6)
MS+Chitosan+GL (n=6)	MS+Chitosan+GL (n=6)	MS+Chitosan+GL (n=6)
MS+GL (n=6)	MS+GL (n=6)	MS+GL (n=6)
MS+Chitosan+IG (n=6)	MS+Chitosan+IG (n=6)	MS+Chitosan+IG (n=6)
MS+IG (n=6)	MS+IG (n=6)	MS+IG (n=6)

MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*

2.3.3. *Collection of samples*

On days 7, 14 and 28 of the study, after anaesthesia with intraperitoneal 3 mg/kg xylazine and 90 mg/kg ketamine HCl, tissue samples were taken from the extracted alveolar bone. Alveolar bone samples were fixed in 10% formaldehyde solution and sent to Muğla Sıtkı Koçman University Faculty of Medicine's Pathology Department for immunohistochemical analysis.

2.4. Immunohistochemical Analysis of Tissues

For histopathological examination, tissues were fixed in 10% neutral buffered formalin, decalcified with 5% EDTA, embedded in paraffin blocks, cut into 4-micron sections, and then stained with haematoxylin and eosin (H&E). After routine microscopic evaluation, 4-micron tissue sections were placed on polylysine coated glass slides (Thermo Scientific) for immunohistochemical staining. Sections were washed in xylene, followed by dehydration in graded ethanol and phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide for 20 minutes. Antigen retrieval was performed using 10% citrate buffer and non-specific background staining was blocked by incubation with TBS for 5 minutes. Osteocalcin (Thermofisher PA5-78871) and SPARC osteonectin (GTX133747 IHC-P) antibodies were applied using the Leica Bond-Max fully automated immunohistochemistry device and incubated with secondary antibodies for 20 minutes. Chromogen 3,3'-diaminobenzidine was also incubated for 5 minutes as a substrate for colour development. Each slide was washed in tap water and counterstained with haematoxylin. Osteoblast and osteocyte levels were assessed by light microscopy (BX46 clinical microscope, Olympus, Tokyo, Japan).

2.5. Semi-quantitative Scoring of Histopathologic Parameters

Haematoxylin eosin-stained specimens were supported by immunohistochemical images and bone formation was assessed by the presence of osteoblasts and osteocytes in the

fibroconnective tissue. The percentage of bone tissue formed along the entire alveolar socket surface after tooth extraction was quantified as a percentage.

2.6. Statistical Analysis

Analyses were performed using IBM SPSS 20 statistical analysis software. Data was presented as mean, standard deviation, median, minimum, maximum, percentage, and number. Normal distribution of continuous variables was analysed using Shapiro-Wilk test, Kolmogorov-Smirnov test, Q-Q plot, and Skewness & Kurtosis. The ANOVA test was used when the normal distribution condition was met, and the Kruskal-Wallis test when it was not met, when comparing continuous variables with more than two independent groups. Post-hoc tests after ANOVA were performed using Tukey's test when the variances were homogeneous and Tamhane's T2 test when the variances were nonhomogeneous. For post-hoc tests after Kruskal-Wallis test, Kruskal-Wallis 1-way ANOVA (k samples) test was used. Pearson chi-squared test was used when the expected value was (>5) and Fisher-Freeman-Halton test when the expected value was (<5) for comparisons greater than 2x2 between categorical variables. Statistical significance was $p<0.05$.

3. FINDINGS

3.1. Histopathological Evaluation

Day 7: Increased congested blood vessels, defective bone fragments and foreign body granulation tissue were seen in 2 cases. Increased fibroblastic activity and marked granulation tissue and, to a lesser extent, osteoblastic cell formation was also observed (Figure 1a).

Day 14: A significant increase in osteoblastic activity was observed with a small amount of congested blood vessels and a limited area of inflammation. Osteocyte and osteoblast development in fibroconnective tissue and up to 50% mature bone formation were observed (Figure 1b).

Day 28: Formation of mature bone trabeculae surrounded by up to 85% osteoblastic cell rim within fibroconnective stromal tissue was evident, vasculature was in the form of normal tissue. No inflammation was observed (Figure 1c).

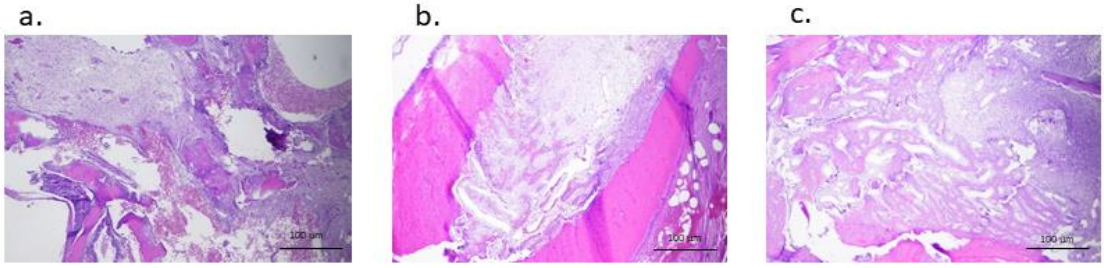

Figure 1. Histopathological evaluation. a. Haematoxylin and eosin staining, original magnification x40 (day 7). Congested blood vessels increased fibroblastic activity and granulation tissue with few osteoblasts. b. Haematoxylin and eosin staining, original magnification x40 (day 14). Reduced congested blood vessels, decreased inflammation, increased osteoblastic activity and initiation of mature bone formation. c. Haematoxylin and eosin staining, original magnification x40 (day 28). Formation of mature bone trabeculae surrounded by a prominent rim of osteoblastic cells.

3.2. Results of Statistical Analysis

According to the results of the study, there was no statistically significant difference in bone formation percentage between both day 7 and 14 groups ($p>0.05$) (Table 2). On day 28, a statistically significant difference was observed between the negative control group and the MS + chitosan + IG group and between the MS group and the MS + chitosan + IG group in terms of bone formation percentage ($p<0.05$) (Table 2). When evaluated in terms of bone formation percentage on different days within the groups, a statistically significant difference was observed in all groups in terms of bone formation percentage on days 7 and 28 ($p<0.05$) (Table 3).

Table 2. Comparing the percentages of bone formation among groups occurring on different days.

		Bone Formation Percentages							
Time	Groups	Mean	Standard Deviation	Median	Minimum	Maximum	Chi-square	*p*	post-hoc
Day 7	Negative Control	2	4	0	0	10	3.165	0.788	
	MS+Chitosan (control)	5	8	0	0	20			
	MS (control)	2	4	0	0	10			
	MS+Chitosan+GL	7	8	5	0	20			
	MS+GL	5	8	0	0	20			
	MS+Chitosan+IG	8	12	5	0	30			
	MS+IG	7	12	0	0	30			
Day 14	Negative Control	15	10	15	0	30	6.793	0.340	
	MS+Chitosan (control)	20	12	20	0	30			
	MS (control)	20	7	20	10	30			
	MS+Chitosan+GL	27	14	30	10	40			
	MS+GL	25	14	25	10	40			
	MS+Chitosan+IG	32	12	30	20	50			
	MS+IG	28	13	20	20	50			
Day 28	Negative Control	44	5	40	40	50	20.607	0.002	(Negative Control-MS+Chitosan+IG), (MS-MS+Chitosan+IG)
	MS+Chitosan (control)	62	15	55	50	80			
	MS (control)	47	8	45	40	60			
	MS+Chitosan+GL	60	11	60	50	80			
	MS+GL	66	9	60	60	80			
	MS+Chitosan+IG	70	9	70	60	80			
	MS+IG	56	9	50	50	70			

(MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*)

Table 3. Comparing the percentages of bone formation on different days within the groups.

Groups	Time		Bone Formation Percentages					Chi-square	*p*	Post-Hoc
			Mean	Standard Deviation	Median	Minimum	Maximum			
Negative Control	Day	7	2	4	0	0	10	13.105	0.001	Day 7-28
		14	15	10	15	0	30			
		28	44	5	40	40	50			
MS+Chitosan	Day	7	5	8	0	0	20	12.913	0.002	Day 7-28
		14	20	12	20	0	30			
		28	62	15	55	50	80			
MS	Day	7	2	4	0	0	10	14.558	0.001	Day 7-28
		14	20	7	20	10	30			
		28	47	8	45	40	60			
MS+Chitosan+GL	Day	7	7	8	5	0	20	13.963	0.001	Day 7-28
		14	27	14	30	10	40			
		28	60	11	60	50	80			
MS+GL	Day	7	5	8	0	0	20	13.069	0.001	Day 7-28
		14	25	14	25	10	40			
		28	66	9	60	60	80			
MS+Chitosan+IG	Day	7	8	12	5	0	30	14.190	0.001	Day 7-28
		14	32	12	30	20	50			
		28	70	9	70	60	80			
MS+IG	Day	7	7	12	0	0	30	11.555	0.003	Day 7-28
		14	28	13	20	20	50			
		28	56	9	50	50	70			

(MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*)

When comparing bone formation on different days within the groups, a statistically significant difference was found between bone formation on different days within the negative control, MS + chitosan, MS, MS + GL and MS + IG groups (p<0.05) (Table 4). Statistical differences in bone formation within groups were found between day 7-28 in the negative control group, day 7-28 in the MS + Chitosan group, day 7-14 and day 7-28 in the MS group, day 7-14 in the MS + GL group and day 7-14 and day 7-28 in the MS + IG group (p<0.05) (Table 4). There was no statistically significant difference between bone formation in the MS + Chitosan + GL and MS + Chitosan + IG groups at day 7, 14 and 28 (p>0.05) (Table 4).

Table 4. Comparing bone formations on different days within the groups.

Groups	Time		Bone Formation				Fisher Freaman Halton	p	Post-Hoc
			Absent		Present				
			Count	Column N %	Count	Column N %			
Negative Control	Day	7	5	83.3%	1	9.1%	8.598	0.012	7-28(day)
		14	1	16.7%	5	45.5%			
		28	0	0.0%	5	45.5%			
MS+Chitosan	Day	7	4	80.0%	2	16.7%	5.934	0.036	7-28(day)
		14	1	20.0%	4	33.3%			
		28	0	0.0%	6	50.0%			
MS	Day	7	5	100.0%	1	8.3%	10.986	0.002	7-14(day). 7-28(day)
		14	0	0.0%	5	41.7%			
		28	0	0.0%	6	50.0%			
MS+Chitosan+GL	Day	7	3	100.0%	3	20.0%	5.205	0.074	
		14	0	0.0%	6	40.0%			
		28	0	0.0%	6	40.0%			
MS+GL	Day	7	4	100.0%	2	15.4%	7.528	0.015	7-14(day)
		14	0	0.0%	6	46.2%			
		28	0	0.0%	5	38.5%			
MS+Chitosan+IG	Day	7	3	100.0%	3	20.0%	5.205	0.074	
		14	0	0.0%	6	40.0%			
		28	0	0.0%	6	40.0%			
MS+IG	Day	7	4	100.0%	2	16.7%	7.031	0.014	7-14(day), 7-28(day)
		14	0	0.0%	5	41.7%			
		28	0	0.0%	5	41.7%			

MS: Microsphere, GL: *G. lucidum*, IG: I.*graveolens*

When the bone formation of the groups on different days was compared, no statistically significant difference was found between the bone formation of the groups on both day 7 and 14 (p>0.05) (Table 5). On day 28, no statistical comparison could be made as bone formation had occurred in all rats.

Table 5. Comparing bone formations within groups occurring on different days.

		Bone Formation					
		Absent		Present			
Time	Groups	Count	Column N %	Count	Column N %	Fisher Freaman Halton	*p*
Day 7	Negative Control	5	17.9%	1	7.1%	3.176	0.924
	MS+Chitosan	4	14.3%	2	14.3%		
	MS	5	17.9%	1	7.1%		
	MS+Chitosan+GL	3	10.7%	3	21.4%		
	MS+GL	4	14.3%	2	14.3%		
	MS+Chitosan+IG	3	10.7%	3	21.4%		
	MS+IG	4	14.3%	2	14.3%		
Day 14	Negative Control	1	50.0%	5	13.5%	5.193	0.709
	MS+Chitosan	1	50.0%	4	10.8%		
	MS	0	0.0%	5	13.5%		
	MS+Chitosan+GL	0	0.0%	6	16.2%		
	MS+GL	0	0.0%	6	16.2%		
	MS+Chitosan+IG	0	0.0%	6	16.2%		
	MS+IG	0	0.0%	5	13.5%		
Day 28	Negative Control	0	0.0%	5	12.8%	NA	NA
	MS+Chitosan	0	0.0%	6	15.4%		
	MS	0	0.0%	6	15.4%		
	MS+Chitosan+GL	0	0.0%	6	15.4%		
	MS+GL	0	0.0%	5	12.8%		
	MS+Chitosan+IG	0	0.0%	6	15.4%		
	MS+IG	0	0.0%	5	12.8%		

(MS: Microsphere, GL: *G. lucidum*, IG: *I. graveolens*)

4. DISCUSSION and CONCLUSION

Vertical and horizontal resorption of alveolar bone after tooth extraction has become an important issue, especially in combination with the need for dental implants. For this reason, the bone healing process that occurs in the alveolar socket has attracted the attention of researchers in recent years. The alveolar socket healing model after tooth extraction in rats allows the evaluation of different osteopromotive products prior to clinical trials, on the alveolar socket bone regeneration that occurs (Koh *et al*., 2018; Kawecki *et al*., 2022; Sukpaita *et al*., 2024).

In our study, an alveolar socket model was created after extraction of the left central maxillary tooth in rats. Different stages of bone healing in the alveolar socket were observed by histopathological examination on the 7th, 14th and 28th day after tooth extraction. Increased fibroblast activity, dense granulation tissue and osteoblast proliferation were observed on day 7 after extraction. Previous studies have reported that fibroblasts accelerate the closure of the socket after extraction and continue to increase until 7 days after extraction (Vieira *et al*., 2015; Hassumi *et al*., 2018). Granulation tissue is formed by fibroblasts that synthesise collagen and extracellular matrix (Luvizuto *et al*., 2010). This granulation tissue then transforms into a provisional matrix. This contains more matrix and fewer inflammatory cells (Araújo et al., 2015). In our study, fibroblasts and granulation tissue decreased and osteoblastic activity increased on day 14. Hanafiah *et al.* (2021) reported that osteoblastic activity, which began to be observed on day 7 following extraction, peaked on day 14. Osteoblasts then form an osteoid matrix and these osteoids mineralise to form woven bone. Osteoblasts arranged on osteoids and

embedded in the matrix become osteocytes. Olaitan *et al*. (2019) reported that the number of osteocytes in the alveolar socket was higher on day 28 after extraction than on previous days. Hassumi *et al.* (2018) reported that mature bone trabeculae, containing osteocytes that fill most of the alveolar socket, were observed on the 28th day after extraction. In our study, mature trabeculae formation was also seen on day 28 in parallel with these studies.

When analysing the bone formation percentage results of our study, the bone formation percentage in the MS + Chitosan + IG group on day 28 was higher than the negative control and MS groups. As in previous studies, the regenerative contributions of chitosan, which is used as a bioactive polymer in bone tissue engineering applications, to bone wound healing is supported in the results of our study (Kowalczyk *et al*., 2021; Guillén-Carvajal *et al*., 2023). There are however no previous studies on the effect of *I. graveolens* on bone regeneration. Therefore, it is difficult to compare this effect of *I. graveolens* on bone regeneration with the available literature data. Ponticelli et al. have reported antibacterial, cytotoxic, and anti-inflammatory effects of *I. graveolens* (Ponticelli *et al*., 2022). The contribution of *I. graveolens* to bone formation together with chitosan may be associated with these existing effects in the literature. The fact that *G. lucidum* did not make a statistically significant difference in the percentage of bone formation may seem to contradict previous studies (Miyamoto *et al*., 2009; Laçin *et al*., 2019). In a study by Laçin *et al*. (2019) on calvarial defects, used together with bone grafting, *G. lucidum* was administered by gastric lavage. Miyamoto *et al.* (2009) administered *G. lucidum* via a diet containing 0.3% ethanol extracts. In our study, topical application of microspheres to the alveolar sockets after extraction without bone grafting differs from the methods used in previous studies. Therefore, different results may be expected. Another result of our study related to the percentage of bone formation is the statistically significant difference between day 7 and day 28 in all groups. The higher percentage of bone formation on day 28 compared to day 7 is in line with previous studies on socket healing after extraction (Okamoto & Russo, 1973; Carvalho *et al*., 1997; Rodrigues *et al.*, 2016).

In our study, bone formation (present, absent) was compared in addition to the percentage of alveolar socket bone formation after extraction. When the comparison of bone formation on different days within the groups was analysed, no statistically significant difference was found between the bone formation on days 7, 14 and 28 in the MS + Chitosan + GL and MS + Chitosan + IG groups. This shows that *G. lucidum* and *I. graveolens* together with chitosan contribute to bone formation from day 7 onwards. In their study, Yadav *et al.* (2021) showed that chitosan scaffolds induce osteogenic morphogenesis for cell adhesion and proliferation, neovascularisation, mineralisation, and bone regeneration in bone tissue engineering. Kim *et al.* (2016) highlighted the need to develop chitosan hydrogel surfaces that provide a suitable osteogenic microenvironment to facilitate osteogenesis with hydrogels. The synergistic effect of chitosan with *I. graveolens* and *G. lucidum* on alveolar bone formation in our study supports these studies.

Previous studies have shown the antimicrobial and anti-inflammatory effects of *G. lucidum* and *I. graveolens*. However, this study demonstrated the potential of these two bioactive agents for bone regeneration in post-extraction alveolar sockets. Further research is needed as this is the first study to evaluate the effect of these two agents on alveolar bone regeneration.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Muğla Sıtkı Koçman University, Animal Experimentation and Research Centre, Local Ethics Committee for Animal Experimentation (MUDEM-HAYDEK). 11.01.2021-19/21.

Authorship Contribution Statement

Ali Batuhan Bayırlı: Conception, Materials, Data collection and processing, Analysis and Interpretation, Writing the Original Draft, and Manuscript Review. **Serhat Sezgin:** Resources, Conception, Analysis and Interpretation, Supervision, and Writing. **Ezgi Eren Belgin:** Materials, Design, Analysis and Interpretation and Writing. **Leyla Tekin:** Analysis and Interpretation and Writing. **Hüseyin Çiçek:** Materials, Analysis and Interpretation, Supervision. **Ayşegül Demirbaş:** Conception, Data collection and processing. **Cankız Gizem Delibalta:** Investigation, and Materials. **Fatma Yılmaz:** Investigation, Supervision, and Validation.

Orcid

Ali Batuhan Bayırlı https://orcid.org/0000-0003-1028-9461
Serhat Sezgin https://orcid.org/0000-0001-7899-8171
Ezgi Eren Belgin https://orcid.org/0000-0002-1089-3741
Leyla Tekin https://orcid.org/0000-0003-1172-5536
Hüseyin Çiçek https://orcid.org/0000-0001-9719-6481
Ayşegül Demirbaş https://orcid.org/0000-0002-9659-2578
Cankız Gizem Delibalta https://orcid.org/0000-0002-3199-3709
Fatma Yılmaz https://orcid.org/0000-0003-1954-6444

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