# Histological Evaluation of the Osteoinduction Capability of Demineralized Human Dentine Implanted into Muscle: A Chronologic Study

**Original Article**

*Shahriar Ahmadpour[1], Amir Khajavi[2] and Khadijeh Foghi[1]*

[1]*Department of Anatomy, Medicine School, North Khorasan University of Medical Sciences, Bojurd, Iran*

[2]*Department of Periodontics, Dentistry School, North Khorasan University of Medical Sciences, Bojurd, Iran*

## ABSTRACT

**Background:** Nowadays different materials are being used in alveolar bone reconstruction surgeries. Dentin is one of the biomaterials has drawn attention in recent years, but the results of studies are inconsistent.

**Aim:** The aim of this study was to investigate osteoinduction capability of demineralized human derived-dentin particles implanted into muscular tissue.

**Material and Methods:** The present experimental study was carried out on adult Rats (8 weeks old ,200-250 gram). Demineralized human dentin (DHD) was implanted (100mg) in latissimus dorsi muscle under the deep anesthesia. At the end of one, second and fourth months, tissue samples were removed and stained with H&E.

**Result:** At the end first month, the particles were enclosed with connective tissue. Multinucleated giant osteoclast attached to the DHD particle, capillaries and differentiating cells in vicinity of particles less than 250 µm were found. Fibroblasts and differentiating cells around the scalloped borders of the large dentin particles (> 850µm) were found. At the end of second month differentiating cells from deeper layer of collagenous capsule, young osteoblast and osteoid deposition adjacent and between the particles (250 µm) were seen. At the end of fourth month capillaries, differentiating cells, osteoid formation and osteoblasts were also found in vicinity of the ruffled margin of the large particles. Young osteoblasts and fibroblasts were observed in dentin niches. Quantification of new bone formation showed that meaningful difference between the 120 days (45±3 $\mu m^2$), 60 days (23±5 $\mu m^2$) and 30 days (0) ($p<0.05$).

**Conclusion:** Our results suggest that demineralized human dentin particles less than 250 µm induce osteoinduction two month after implantation. Larger particles (>850µm) showed limited osteoinduction only in ruffled margins and dentin artifactal niches after four months. Additionally bone formation proceeded at higher rate after 120 days.

**Received:** 13 March 2021, **Accepted:** 03 May 2021

**Key Words:** Demineralized, dentin, muscle, osteoinduction.

**Corresponding Author:** Shahriar Ahmadpour, PhD, Department of Anatomy, Medicine School, North Khorasan University of Medical Sciences, Bojurd, Iran, **Tel.**: +98 (58) 31518 82231, **E-mail:** Shahahmadpour@gmail.com

**ISSN:** 1110-0559, Vol. 45, No.2

## INTRODUCTION

Nowadays different biomaterials are being used in the reconstructive field of medicine and dentistry. Nevertheless, the repair of bone resulting from trauma or developmental craniofacial anomalies has remained as a challenge for regenerative surgeries[1,2]. The matrix of dentin due to osteogenic and chemotactic potentials has been studied as bone substitute[3]. Previous studies have shown that osteoinductivity property of the grafting material depends on many factors including demineralization. It has been proposed that demineralized dentin matrix (DDM) is a more effective bone-inducing matrix than calcified dentin matrix owing to possible inhibitory effects of apatite salts on releasing of bone inducing factors such as bone morphogenic proteins (BMPs)[4]. Despite the importance of the demineralizing processes on exposing the bone inducing factors, authors have reported different results and, in some cases, controversial. For instance, Koga *et al* have recently recommended the use of 1000-µm particles, following partial demineralization with 2% $HNO_3$, compared to non-demineralized or completely demineralized dentin[5]. Togari *et al* demonstrated that dentin particles 250–500 µm in size were highly efficient in osteoinduction[6]. Several studies using transplantation of human dentin blocks (5–6 mm in diameter) for rabbit bone defects showed progressive dentin-bone ankylosis after 3 months, with osseous replacement resorption and no sign of inflammation[7,8]. Another study reported no significant osteoinduction with dentin size particle 350-500 µm[9]. It has been shown that the success of a dental implant is dependent on a variety of factors including chemical, physical, mechanical, and topographic characteristics of its surface. The surface properties of the

 *DOI: 10.21608/ejh.2021.67569.1446*

implant (biomaterials) has been shown to influence the differentiation and proliferation of bone forming cells, osteoblast, and up –regulation of transcription factors involving bone formation and shorter healing times[10]. Thus far most studies have deal with the particle size of the tooth-derived materials and given the discrepancies in the literatures, we aimed to answer this question: could the different results be attributed to the surface geometry or topographic characteristics of the dentin particles? In this study human demineralized dentin was implanted in the rat latissimus dorsi muscle and the rate of osteogenesis was assessed chronologically.

## MATERIALS AND METHODS

This study was carried out on male Wistar rats (age 8weeks, body weight 200–250 g, n=15). All rats maintained in animal house and allowed free access to drinking water and standard rodent diet. Experiments performed during the light period of cycle and conducted in accordance with Regional Committee of Ethic complied with the regulations of the European Convention on Vertebrate Animals Protection (2005)[9].

### *Human Dentin Processing*

The protocol of the tooth collecting was approved by the ethics committee of the North Khorasan University of Medical Sciences (NKUMS). Signed consent was given by patients after a thorough explanation regarding the study. Teeth were free of carious lesion. Extracted teeth from patients with metabolic disorders were excluded. Their roots were sectioned, cleaned from any pulp or ligament residues and were immediately demineralized in according to the protocol as briefly as follow[4,5]:

1. Dentin particles were demineralized in formic acid 5% (changed every 72 hours) at room temperature for 35 days
2. The decalcification time for each size of dentin particles was determined by measuring the concentration of eluted Ca in solution and residual Ca in the dentin over time using Calcium E test kit (Wako, Japan). Then, each DDM particle was extensively rinsed twice in 0.1 M Tris-HCl (pH 7.4), for 10 minutes.
3. crushing with a percussion mill. The particles were collected and collectively trimmed as closely as possible to 200-1000 µm using a calibrated mesh filter, finally were washed thoroughly in 1.0 M sodium chloride[4,5].

### *Demineralized human dentin (DHD) implant*

The study was performed at educational and Research center for biomedical lab. The animals were anesthetized with a 3mL intraperitoneal solution containing a mixture of Ketamine (25 mg/kg; Parke-Davis, Morris Plains, NJ, USA) with acepromazin (0.1 mg/kg; Ingelheim Vetmedica Inc., St. Joseph, MO, USA)[11,12]. The surgical field was shaved and disinfected with povidone-iodine 10%. A mid-sagittal incision through the skin was made after local infiltration of 2% lidocaine hydrochloride with 1: 100,000 epinephrine 1.8mL. The latissimus dorsi muscle was carefully exposed, a muscular pouch (10mm) was made and filled with 100mg demineralized dentin. Antimicrobial prophylaxis (cephalosporin 15 mg/kg, twice a day) was continued for 48 hours after surgery[11,12]. At the end of first, second and fourth month the implanted tissues were harvested under deep anesthesia and kept in formalin 10%. The samples were processed by routine histological method and stained with H&E. The samples were then examined with an optical microscope at 40× magnification (Nikon Eclipse E400; Nikon, Tokyo, Japan) that was linked via a digital camera (Nikon Fuji HC-300 ZI; Nikon) to a personal computer.

### *Quantification of new bone*

For quantification of the new bone formation, a simple morphometric analysis was used; tissues of the specimens were divided into two compartments: newly formed bone and connective tissue. A stereological frame was superimposed on each randomized field. A stereological frame was superimposed on each photograph; the hit points or intersections for each phase (interested phase.e.g. new bone formation) were counted and recorded. Using this method, the proportions of the histological phases (new bone and other histologic phases) were obtained for each studied field. The surface area (SA) of each counting frame (150×120 µm) was 18,000 $\mu m^2$. Finally, the number of hit of points was inserted into the following formula:

$$SA=I \text{ phase } A/Pn$$

where SA (surface area) indicates the proportion of a specific tissue (e.g. New bone formation), I indicates the number of intersections for interested phase (A in this case represents new bone formation) and Pn represents total number of stereological frame. As mentioned earlier, the surface ratios for each section were calculated[9].

### *Statistical analysis*

All data are expressed as mean±standard deviation. The comparison was made by paired ANOVA, and *P-values*<0.05 were considered statistically significant. Calculations were performed using the SPSS Statistics version 17.0 software (SPSS Inc., Chicago, IL, USA)

## RESULTS

### *Implant at 30 days*

The DHD particle were enclosed with connective tissue. Blood vessels were seen between the enclosed particles (Figure 1). Multinucleated giant osteoclast attached to the DHD particle, capillaries and differentiating cells in vicinity of particles less than 250 µm were found (Figure 2). Additionally, fibroblasts and differentiating cells around the scalloped borders of the large DHD particles were found (Figure 3).

### *Implant at 60 days*

More connective tissue, blood vessels and collagen fibers deposition in particular in scalloped margins of large DHD particles were formed (Figure 4). In plain margins of DHD particles attached differentiating cells surrounded with dilated venules were found (Figure 5). Small DHD particles were enclosed with multilayers of collagen fibers. Differentiating cells from deeper layer of collagenous capsule, young osteoblast and osteoid deposition adjacent and between the DHD particles were seen (Figure 6).

### *Implants at 120 days*

Presence of more connective tissue, collagen fibers and numerous capillaries in the ruffled borders of the large DHD (>800 µm) were the dominant microscopic findings (Figure 7). In some regions capillaries, differentiating cells, osteoid formation and osteoblasts were found in vicinity of the ruffled margin of the large DHD (Figure 8). Young osteoblasts and fibroblasts were observed in dentin niches (artifactual cleft) (Figure 9).

Quantification of bone formation: The ratio of SA (bone) to total area of each probe was assessed and was 45±3 $\mu m^2$ for the 120 days ,23±5 $\mu m^2$ for the 60 days and non-significant for the 30 days. The comparison between the groups showed significant level of difference ($P$<0.05).

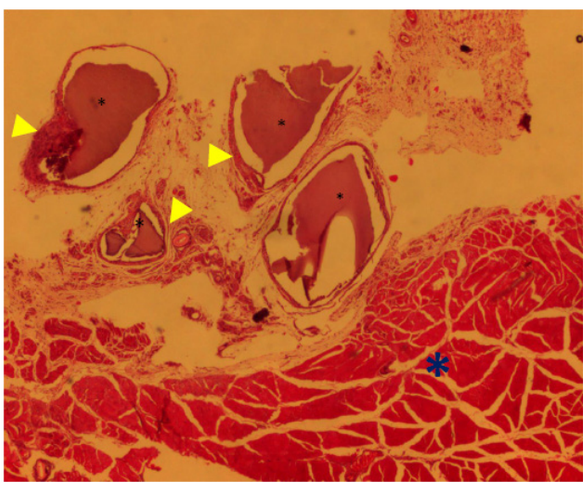

**Fig. 1:** The DHD particles (stars) implanted in muscular (blue star) tissue after one month. The DHD particles are surrounded by connective tissue (yellow arrow heads)

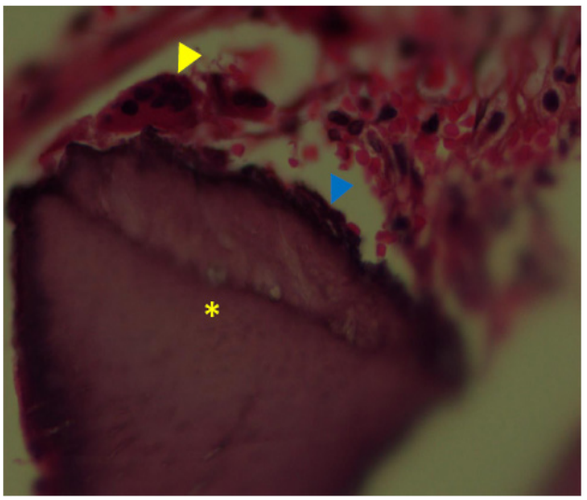

**Fig. 2:** A DHD particle after one month. Multinucleated giant osteoclast (yellow arrowhead) attached to the particle. Vessels invasion and differentiating cells ( blue arrowhead) are seen in the vicinity of the DHD(yellow star). X100

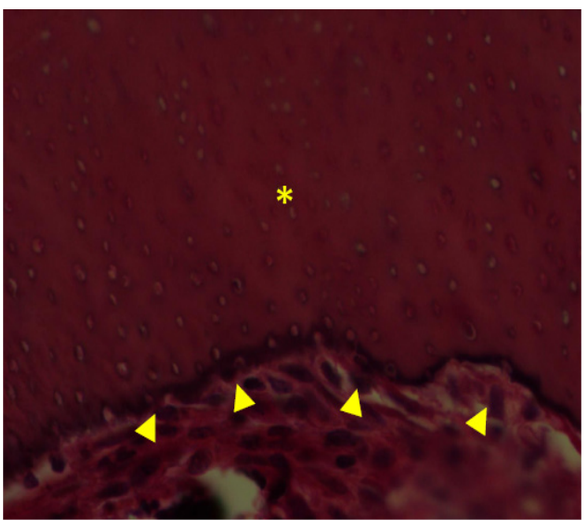

**Fig. 3:** A DHD particle(>800µm) after one month. Connective tissue surrounding the DHD particle (yellow star) and differentiating cells (yellow arrowheads) attached to the border of the particle are seen. X40

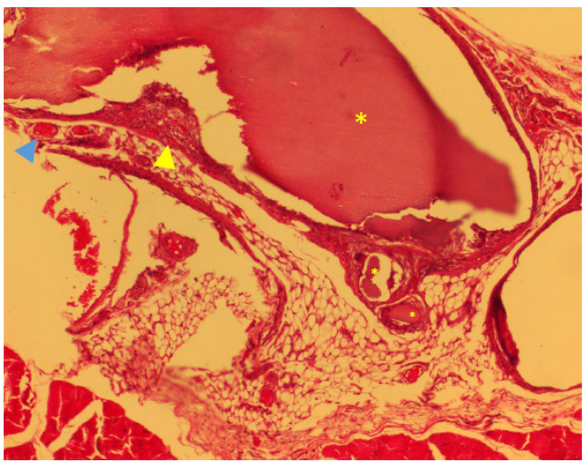

**Fig. 4:** DHD particles implanted in muscular tissue after two months. Implanted particles( yellow stars) are surrounded by surrounded by connective tissue (yellow arrowhead) and blood vessels(blue arrowhead). X20

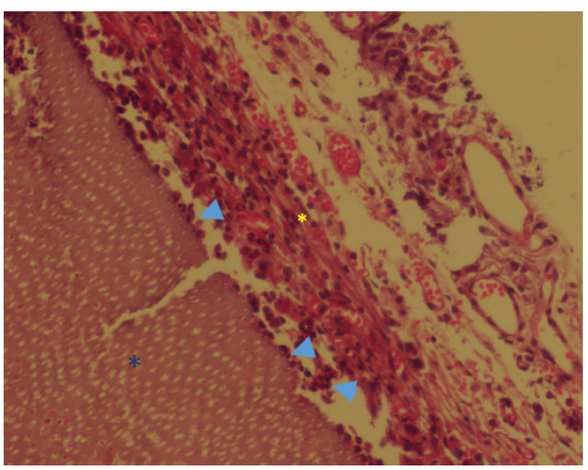

**Fig. 5:** A DHD particle(>800µm) after two months. Connective tissue in vicinity (yellow star) the DHD particle(blue star)and differentiating cells (blue arrowheads) attached to the border of the particle are seen. X40

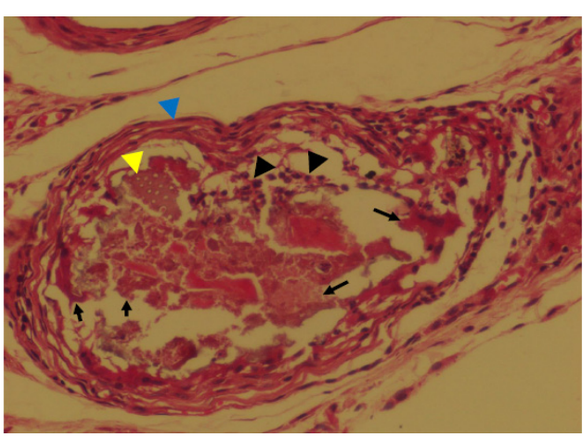

**Fig. 6:** Small DHD (yellow arrowhead) particle is enclosed with collagen fibers (blue arrowhead). Deposition of osteoid (black arrows) and differentiating cells (black arrowheads). H&E staining .X40

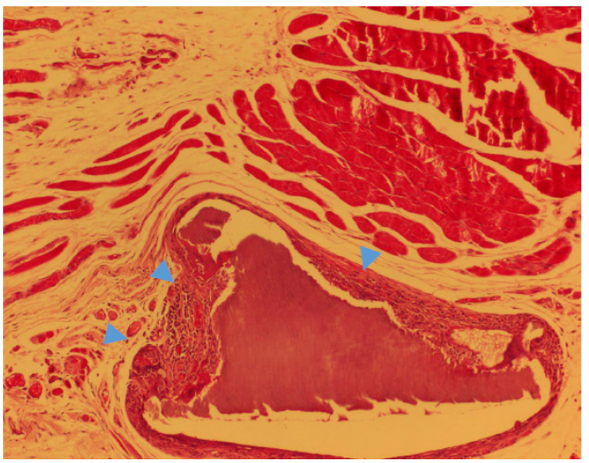

**Fig. 7:** DHD particle after 4 months. Raffled borders of the particle invaded by the connective tissue and blood vessels(blue arrowheads).X20

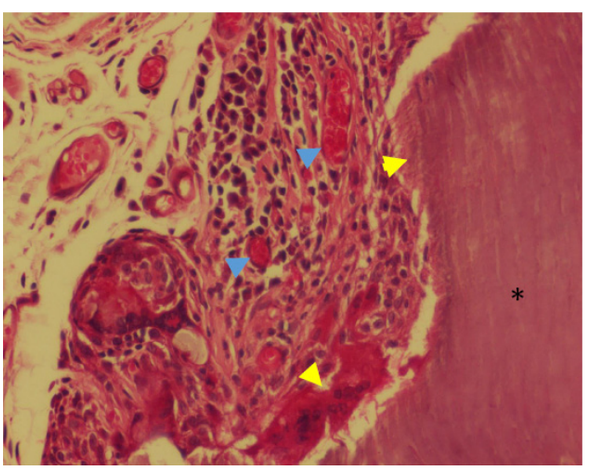

**Fig. 8:** numerous capillaries(blue arrow heads) invaded the connective tissue in vicinity of DHD particle(star) after four months. Differentiating cells, osteoblasts and osteoid (yellow arrowhead) deposition are seen.X40

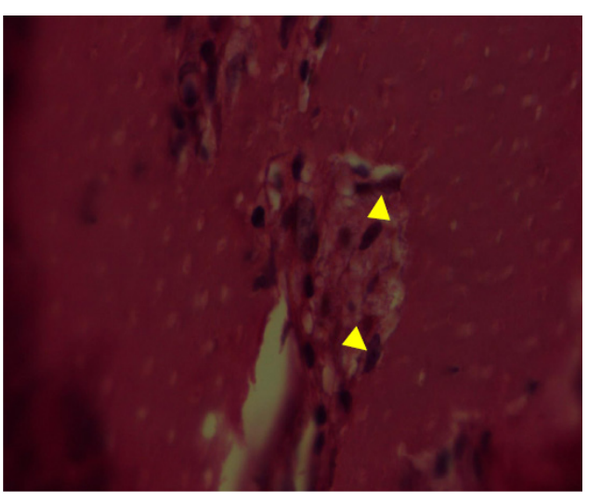

**Fig. 9:** A dentine artifactual niche and differentiating cells (yellow arrowheads) attached to the DHD raffled border after four months. H&E staining. X100

## DISCUSSION

This animal study was carried out to examine the relation between the geometry, interface, particle size and the rate of osteogenesis of implanted demineralized human dentin (DHD) in muscular tissue. The results showed that the application of DHD induced limited effects on new bone formation before 2 months, as the collagen fibers deposition, cell differentiation and vessels invasion around the implanted particles were the dominant histological features. Another set of our findings revealed that osteoinduction process is induced around the particle sized less than 250 µm while in contrast limited osteoinduction were observed around the ruffled (scalloped) borders of the particles up to 800 µm. The results of morphometric analysis also revealed that bone formation proceeded at higher rate after 120 days. According to these findings osteoinduction and osteoconduction processes depend on the size, surface topography of the DHD particles and time. Connective tissue formation around the particles, neovascularization, cell differentiation and new bone formation by intramembranous ossification were the major microscopic events, but the manner of cell differentiation showed different pattern in the large and small DHD particles. Cellular differentiation and migration occurred from periphery, deep layer of collagenous envelop, toward the small sized particle (centripetal manner), while the large particles provided an anchoring surface to the differentiating cells. These findings are in line with the results of Kim *et al*. They showed that osteogenesis typically begins with fibrous tissue and vessels formation after two months[14]. These findings indicate the particle size along with the presence of bony niches as a surface geometric factor play a pivotal role in osteoinductive properties of the implanted biomaterials[13]. Precisely microscopic findings showed that particles sized between >250 µm failed to induce osteoinduction process even after four months and limited osteogenesis were only induced in the ruffled margins of DHD. Previously reported results on the particle size and osteoinductivity are to some extent contradictory. Togari *et al* reported the optimum results by particle sized between250-500 µm[5] and Koga *et al* reported new bone formation by using particle sized 1000 µm[6]. Dentin tissue contains chemotactic and growth factors trapped in during mineralization. Demineralization could release or expose the trapped growth factor such as BMPs[15]. Biochemically dentin is a complex structure and consists of BMPs and amelogenin peptide[16]. Chemotactic properties of the released factor by demineralization and surface geometry of the dentin particle, interface, make the DHD particles as a suitable surface for anchoring and attachment of undifferentiated mesenchymal cells and new bone formation in intramembranous manner[12,17,18]. Given the results of this study it seems the smaller particle size could induce osteoinduction by exposing the growth factors and subsequently neovasculogenesis, recruitment and differentiation of mesenchymal cells to bone forming cells[18,19]. One of the methods to examine the osteoinduction potency of biomaterials and their biologic behaviors in the non –osseous host tissue such as muscular tissue that osteogenesis don't occur naturally[11,12,20]. In this study DHD particles were implanted in the latissimus dorsi, therefore by combination of the particle geometry along with the vascularity and distensibility of the muscular tissue could provide cell differentiation, contact, attachment and sufficient blood supply for osteoinduction process[20,21]. Neovasculogenesis plays an important role in the bone healing and formation osteproginator cells differentiate in vicinity of the blood vessels. Additionally, pericytes wrap around the endothelial cells could differentiate to pre-osteoblasts, assisting new bone formation[22,23]. Therefore, host tissue factors including vascularity affects the subsequent response of the implanted materials. The behavior of the implanted small and large particles showed different patterns. Osteoinduction by the large DHD would not happen less than 4 months but the rate of new formation through osteoinduction process was not significant. Mesenchymal cells first established a physical contact with surface of the large DHD, differentiating and subsequently new bone formed after four months. In case of the small DHD, mesenchymal cells apparently migrate from collagenous sac enclosing the particles in a centripetal manner, differentiate to blast cells without attachment to the particles and produce osteoid-liked matrix after two months.

## CONCLUSION

It can be concluded that size of the dentin particles, 3-dimentional ruffled margin as a surface geometric property and connective tissue capsule are the main players of the osteoinduction process through mesenchymal cell recruitment, attachment and differentiation.

## CONFLICT OF INTERESTS

There are no conflicts of interest.

## REFERENCES

1. Zizzari VL, Zara S, Tetè G, Vinci R, Gherlone E, Cataldi A. Biologic and clinical aspects of integration of different bone substitutes in oral surgery: a literature review. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016; Oct;122(4):392-402. doi: 10.1016/j.oooo.2016.04.010. Epub 2016 May 12. PMID: 27496576.
2. Agrali OB, Yildirim S, Ozener HO, Köse KN, Ozbeyli D, Soluk-Tekkesin M, Kuru L. Evaluation of the Effectiveness of Esterified Hyaluronic Acid Fibers on Bone Regeneration in Rat Calvarial Defects. Biomed Res Int.2018; Jun 28;2018:3874131. doi: 10.1155/2018/3874131. PMID: 30050929; PMCID: PMC6046155.
3. Okamoto M, Takahashi Y, Komichi S, Cooper PR, Hayashi M. Dentinogenic effects of extracted dentin matrix components digested with matrix metalloproteinases. Sci Rep. 2018 Jul 16;8(1):10690. doi: 10.1038/s41598-018-29112-3. Erratum in: Sci Rep( 2020 );Sep 15;10(1):15342. PMID: 30013085; PMCID: PMC6048071.

4. Li R, Guo W, Yang B, Guo L, Sheng L, Chen G, Li Y, Zou Q, Xie D, An X, Chen Y, Tian W. Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration. Biomaterials. 2011 ; 32(20):4525-38. doi: 10.1016/j.biomaterials(2011);03.008. Epub 2011 Mar 31. PMID

5. Koga T, Minamizato T, Kawai Y, Miura K, I T, Nakatani Y, Sumita Y, Asahina I. Bone Regeneration Using Dentin Matrix Depends on the Degree of Demineralization and Particle Size. PLoS One. 2016;Jan 21;11(1):e0147235. doi: 10.1371/journal.pone.0147235. PMID: 26795024; PMCID: PMC4721666.

6. Togari K, Miyazawa K, Yagihashi K, Tabuchi M, Maeda H, Kawai T, *et al*. Bone regeneration by demineralized dentin matrix in skull defects of rats." Journal of Hard Tissue Biology.2012; 21(1): 25-34

7. Andersson L, Ramzi A, Joseph B. Studies on dentin grafts to bone defects in rabbit tibia and mandible; development of an experimental model. Dent Traumatol. 2009; Feb;25(1):78-83. doi: 10.1111/j.1600-9657.2008.00703.x. PMID: 19208015.

8. Andersson L. Dentin xenografts to experimental bone defects in rabbit tibia are ankylosed and undergo osseous replacement. Dent Traumatol. 2010;Oct;26(5):398-402. doi: 10.1111/j.1600-9657.2010.00912.x. Erratum in: Dent Traumatol. 2010; Dec;26(6):532. PMID: 20636361.

9. Kadkhodazadeh M, Ghasemianpour M, Soltanian N, Sultanian GR, Ahmadpour S, Amid R. Effects of fresh mineralized dentin and cementum on socket healing: a preliminary study in dogs. J Korean Assoc Oral Maxillofac Surg (2015); Jun;41(3):119-23. doi: 10.5125/jkaoms.2015;41.3.119. Epub 2015 Jun 23. PMID: 26131427; PMCID: PMC4483524.-

10. Lerner UH, Kindstedt E, Lundberg P. The critical interplay between bone resorbing and bone forming cells. J Clin Periodontol. 2019; Jun;46 Suppl 21:33-51. doi: 10.1111/jcpe.13051. PMID: 30623989.

11. Nam JW, Kim MY, Han SJ. Cranial bone regeneration according to different particle sizes and densities of demineralized dentin matrix in the rabbit model. Maxillofac Plast Reconstr Surg. 2016; Jul 5;38(1):27. doi: 10.1186/s40902-016-0073-1. PMID: 27441186; PMCID: PMC4932123.

12. Reis-Filho CR, Silva ER, Martins AB, Pessoa FF, Gomes PV, de Araújo MS, Miziara MN, Alves JB. Demineralised human dentin matrix stimulates the expression of VEGF and accelerates the bone repair in tooth sockets of rats. Arch Oral Biol. 2012; May;57(5):469-76. doi: 10.1016/j.archoralbio.2011.10.011. Epub 2011 Oct 29. PMID: 22041019.

13. AlFarraj Aldosari A, Anil S, Alasqah M, Al Wazzan KA, Al Jetaily SA, Jansen JA. The influence of implant geometry and surface composition on bone response. Clin Oral Implants Res .2014; Apr;25(4):500-5. doi: 10.1111/clr.12190. Epub 2013 May 30. PMID: 23718131.

14. Kim YK, Kim SG, Bae JH, Um IW, Oh JS, Jeong KI. Guided bone regeneration using autogenous tooth bone graft in implant therapy: case series. Implant Dent.2014; Apr;23(2):138-43. doi: 10.1097/ID.0000000000000046. PMID: 24637527.

15. Glowacki J. Demineralized Bone and BMPs: Basic Science and Clinical Utility. J Oral Maxillofac Surg. 2015; Dec;73(12 Suppl):S126-31. doi: 10.1016/j.joms.2015.04.009. PMID: 26608140.

16. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. Front Biosci (Elite Ed). 2011; Jan 1; 3:711-35. doi: 10.2741/e281. PMID: 21196346; PMCID: PMC3360947.

17. Estrela C, Alencar AH, Kitten GT, Vencio EF, Gava E. Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. Braz Dent J. 2011;22(2):91-8. doi: 10.1590/s0103-64402011000200001. PMID: 21537580.

18. Chung SH, Heo SJ, Koak JY, Kim SK, Lee JB, Han JS, Han CH, Rhyu IC, Lee SJ. Effects of implant geometry and surface treatment on osseointegration after functional loading: a dog study. J Oral Rehabil. 2008; Mar;35(3):229-36. doi: 10.1111/j.1365-2842.2006.01653.x. PMID: 18254802.

19. Tamari T, Kawar-Jaraisy R, Doppelt O, Giladi B, Sabbah N, Zigdon-Giladi H. The Paracrine Role of Endothelial Cells in Bone Formation via CXCR4/SDF-1 Pathway. Cells. 2020; May 26;9(6):1325. doi: 10.3390/cells9061325. PMID: 32466427; PMCID: PMC7349013.

20. Albrektsson T, Johansson C. Osteoinduction, osteoconduction and osseointegration. Eur Spine J.2001; Oct;10 Suppl 2(Suppl 2):S96-101. doi: 10.1007/s005860100282. PMID: 11716023; PMCID: PMC3611551.

21. Feller L, Chandran R, Khammissa RA, Meyerov R, Jadwat Y, Bouckaert M, Schechter I, Lemmer J. Osseointegration: biological events in relation to characteristics of the implant surface. SADJ. 2014; Apr;69(3):112, 114-7. PMID: 24974481.

22. Genova T, Petrillo S, Zicola E, Roato I, Ferracini R, Tolosano E, Altruda F, Carossa S, Mussano F, Munaron L. The Crosstalk Between Osteodifferentiating Stem Cells and Endothelial Cells Promotes Angiogenesis and Bone Formation. Front Physiol. 2019; Oct 14;10:1291. doi: 10.3389/fphys.2019.01291. PMID: 31681005; PMCID: PMC6802576.

23. Birbrair A, Zhang T, Wang ZM, Messi ML, Mintz A, Delbono O. Pericytes: multitasking cells in the regeneration of injured, diseased, and aged skeletal muscle. Front Aging Neurosci. 2014; Sep 18;6:245. doi: 10.3389/fnagi.2014.00245. PMID: 25278877; PMCID: PMC4166895.