Establishment and Characterization of Rabbit Model for Alveolar Bone Regeneration

Thuy-Duong Thi Nguyen[1], My-Huong Le[1], Tan-Tai Tran[1], Tae-Sung Bae[2], Tung Nguyen-Thanh[3,4]*

1. Odonto-stomatology Faculty, Hue University of Medicine and Pharmacy, Hue University, Hue 49000, Vietnam.
2. Department of Dental Biomaterials, School of Dentistry, Jeonbuk National University, Jeonju 561-756, South Korea.
3. Faculty of Basic Science, Hue University of Medicine and Pharmacy, Hue University, Hue 49000, Vietnam.
4. Institute of Biomedicine, Hue University of Medicine and Pharmacy, Hue University, Hue 49000, Vietnam.

Abstract

The target of this study was to establish an animal model for reconstructing critical-sized alveolar defects, allow the experimental testing of advanced alveolar regeneration without causing any damage to the health of the animal.

Alveolar bone defect operation was executed at the lateral side of the right central incisor to create a bony deformity after tooth extraction in New Zealand White rabbit. They were sacrificed at the fixed point of time to either harvest the skulls or perform Radiographic analysis and histological survey with Hematoxylin and Eosin staining to evaluate the bone regeneration at operative site.

Clinical examination and imaging assessment was capable of presenting a critical-sized alveolar bone defect. Soft tissue started to heal gradually, no signs of inflammation and swelling at surgical site was reported and bone regeneration completed after 8 weeks. Radiographic survey displayed the increasing density contrast after surgery, replaced the C-shaped radiolucent at the surgical site. It also correspond the developement of bone in the harvested skulls, in which the amount of bone started to rise from 0-8 weeks. Histological analysis showed the step-by-step formation of different structures at surgical sites.. The bone and connective tissue completely filled the defect after 8 weeks.

Our surgical procedure was capable of establishing an alveolar defect model in the central incisor region of the rabbit maxilla. It demonstrates the capability of this model as a test bed for assessing the healing capacity of soft tissues and alveolar bone regeneration.

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Introduction

Alveolar bone resorption results from untreated dental bone defects, which happens for many reasons. They may be congenital deformities (alveolar cleft) or pathological such as periodontitis, trauma, tumor removal, operation, tooth extraction, osteoporosis or arthritis [1]. Cleft, lip and alveolar cleft, accounting for almost 1/3 of all congenital abnormalities, are the most common inborn deformities [2]. Next to congenital bony deficiency, periodontitis is an inflammatory disease which can result in the continuous annihilation of bone and connective tissues, leading to tooth loss and ending up with the destruction of alveolar bone [3]. Meanwhile, adequate alveolar bone volume and supportive structure of the alveolar edge are fundamental to get a perfect esthetic and functional reconstruction and manage the adherence of the abutment after implant insertion [4].

Lots of effort has been made to treat the alveolar bone defect by using autologous bone harvested from different parts of the body such as rib, iliac crest or tibia due for easy procedures, large amount of cancellous bone obtained; however, it extends and aggravates postoperative process and leaves a potential disturbance of development of the ilium in the young [5]. Therefore, it is necessary to look for a replacement for autogenous bone.

Continued advances in tissue engineering

Corresponding author:
Tung Nguyen-Thanh, Ph.D
Institute of Biomedicine; Faculty of Basic Science, Hue University of Medicine and Pharmacy, Hue University, Hue 47000, Vietnam.
E-mail: nguyenthanhtung@hueuni.edu.vn; nttung@huemed-univ.edu.vn

have made an enormous progress in the field of bone regeneration including stem cells-based therapy, growth factors combined with biomaterials. Many biomaterials have been developed to overcome the shortcomings of autogenous bone and have achieved optimistic results using bone marrow, platelet, beta tricalcium phosphate (β-TCP), hydroxyapatite etc. [6], [7], [8]. Calcium phosphate - a key representative of biomaterials, has been proved of their biocompability, bioactivity and osteo-conductivity through many different researches [9]. Meanwhile, tissue engineering customarily uses one bioactive operator with key regenerative functions, which are a mixture of activated molecules and growth factors to stimulate cells [10]. A promising approach has been tested recently is the application of the osteo-inductive growth factors such as bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (protein therapy), which have been evaluated and gained remarkable success in clinical practice and research [11]. However, using only growth factors makes the stimulation become oversimplified and insufficient, and probably turns into the adverse effects [12]. Within the past few years, stem cell–based treatment has been broadly examined to prove the regenerative potential. Being known as playing a critical role in cell proliferation and differentiate, many products of stem cells have been used: dental pulp stem cells, bone marrow mononuclear cells, platelet concentrates such as platelet-rich firin (PRF), plate-rich plasma (PRP) [7], [8]. These promise to pave a new path for tissue engineering, especially in bone regeneration.

Many animal models have been developed to further discover the potential of biomaterials in bone regeneration and overcome the limitations of in vitro models in controlling the complexity in the interactions between cells, growth factors, scaffold materials, etc. [13]. The goal of animal models is to test the capacity of forming new bone of the material, avoid adverse tissue response and minimize bone resorption. Rabbits are the biggest animals in the small-sized category. They are non-aggressive, easy to house, handle, observe, reproducible and big enough to bear the trauma of the surgery and anesthetization. Literature has reported some points in common when compared the bone mineral density, fracture firmness between rabbits and human being. Rabbits also demonstrate the remarkable bony metabolism and increased bone circulation rate, mostly cortical remodeling when compared to primates and other rodents. [14]. Therefore, a rabbit model is relevant to imitate an alveolar bone defect in human.

The aim of this study is to develop a model on New Zealand White rabbits (*Oryctolagus cuniculus*) to present a critical-sized alveolar defect and apply the materials right after tooth extraction, which is similar to human alveolar bone defects in case of immediate implant after tooth extraction, periodontitis, etc.

Materials and methods

Animals and housing

30 New Zealand White Rabbits, at least 6-8 weeks old and weighing 2 - 2.5 kg were operated following the standardized procedure which was approved by animal ethical committee of Hue University of Medicine and Pharmacy (Certificate No. H2019/069). All rabbits were housed at the animal experimentation laboratory which room temperature and humidity were maintained at about 25^{o}C and 56%, respectively. A 12-hour light/dark schedule was used. Rabbits were given standard laboratory food pellets and water ad libitum. The rabbits were divided into 0-, 2-, 4-, 6-, 8-week postoperative sacrifice point of time.

Rabbit skull osteological survey

Rabbits were sacrificed by lethal dose of ketamine injection (600 mg). Firstly, scalpel and blades, forceps, scissors were used to expose the bones by cutting out the skin and muscle and removing all the adipose tissue and organs. The bones of the rabbit were bubbled in 3% solution of soda water for 1 hour to eliminate muscles and related structures effectively. Then it took 2 hours for cooling and 3 hours for cleaning following the concept of Baker et al. (2003) and Van Cleave (2010). After that, the remaining body tissue on the bones were rubbed by BP handle and blade as much as possible. Lastly, they were soaked into 10% bleaching water solution for 2 hours to prevent further annihilation by microorganisms and then dried in the sun for 10 hours.

The rabbit skulls were then used for observation of the significant anatomical landmarks, especially the central incisor and the

alveolar bone at the distobuccal of the tooth.

Alveolar bone defect model creation surgery

30 New Zealand White Rabbits, at least 6-8 weeks old and weighing 2-2.5 kg were operated following the standardized procedure which was approved by animal ethical committee of Hue University of Medicine and Pharmacy. 6 rabbits were used for histological survey and 6 were used to harvest the skulls. These two groups were further subdivided into 0-, 2-, 4-, 6-, 8-weeks post-operatively, it was also the sacrificing point of time, namely D0, 2W, 4W, 6W and 8W, respectively.

30 minutes prior to surgery, each animal received an intramuscular injection of 1ml atropine sulfate which acts as preanesthetic agents to promote the effectiveness of anesthesia and 0.5ml Tiletamine (Zoletil 50mg) 15 minutes afterwards. Rabbits were put in a quiet and dark place to get into sedation more easily. The perimandibular and cervical areas were subsequently scrubbed with Povidine (2% chlorhexidine gluconate). Three milliliters of Xylocaine (Lidocaine HCl with 1:100,000 epinephrine) were injected subcutaneously along the incision area to provide additionally local anesthesia and reduce the volume of blood lost. A linear incision around the marginal gingiva, extending to the distal-buccal aspect of the central incisor, along the curvature of the tooth was performed, soft tissues and periodontal ligament were to expose the maxillary alveolus of the central incisor. Subsequently, a carbide bur with a low-speed hand piece was used to create the osteotomy traversing through the lateral bony cortex, tooth roots, and trabecular bone along with saline irrigation.

Tooth root was gently luxated with a root tip elevator and eventually removed with a dental forcep. The oral mucosa was sutured with 5-0 absorbable Vicryl suture. The rabbits were fed with soft diet and injected antibiotics in 3 days. The rabbits were cared of accordingly per protocol and watched by veterinarian until the sacrifice point of time.

Radiographic analysis

Rabbits were taken X-ray immediately after surgery and postoperatively at 2, 4, 6, 8 weeks by X-ray. Images were analyzed using EZDent biomedical software.

Histological procedures

At the end point (0, 2, 4, 6, 8 weeks) rabbits were intravenously injected with air. Maxillae were dissected and stored in 10% paraformaldehyde. Then, samples were washed with phosphate buffered saline (PBS) and immersed in 10% Disodium Ethylene Diamine Tetra Acetic acid (EDTA) for 8 weeks to decalcification. Samples were sectioned and stained with Hematoxylin and eosin (H&E) stain to assess bone formation at the defect site.

Measurement of bone formation

The amount of bone formation was measured by calculating the difference between the initial size of bone defect from the size of the bone defect at 2 weeks, 4 weeks, 6 weeks and 8 weeks after surgery. Both radiographic and histological evaluation was done to estimate the newly formed bone.

Radiographic density were ranked following the scoring method of Miloro et al.; Score 1: no newly formed bone; Score 2: contrast less than contiguous cancellous bone; Score 3: contrast is equal or higher than contiguous cancellous bone, but less than cortical bone; Score 4: contrast is higher than contiguous cortical bone [15]. Bone regeneration was compared according to experimental time (2, 4, 6 and 8 weeks).

Histological analysis was performed using the scoring method of Han et al., based on the percentage of newly formed bone and connective tissue in the defect area. The appearance of bone at the defect as the replacement of connective tissues was the signal of bone regeneration. Each sample was rated on a scale of 0-10. Samples were read separately by 2 pathologists at 2 different point of times [16].

Statistical analysis

The amount of bone formation at 2 weeks, 4 weeks, 6 weeks and 8 weeks after surgery were compared using a Mann-Whitney test. All data were expressed as Mean± SEM. A value of $p \leq 0.05$ was considered to be statistically significant.

Results

Characterization of alveolar defect model

The normal and the alveolar defect-created rabbit skull were visually inspected to uncover that the anterior maxilla held a pair of central incisors and a pair of accessory palatal incisor. The central incisor appeared protrusive while the accessory palatal incisor was far smaller and approximately half of it. The alveolar

socket of an extracted central incisor formed a pocket-like cavity with a dimension of 7–8 mm made it an ideal model for alveolar defect studies which is similar to the alveolar defect after extraction in human. Frontal and lateral view of the skulls revealed the position and the depth of the alveolar needed to be removed to extract the central incisor. (Figure 1)

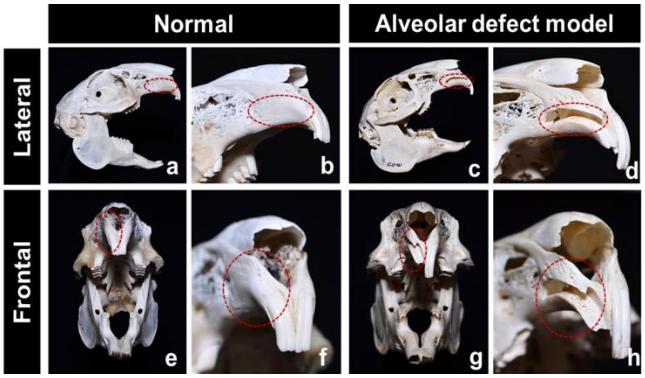


Figure 1. Morphology characteristics of the alveolar bone defect model showing the depth and the orientation of the extraction socket
a, b, c, d. Lateral aspect of a harvested intact rabbit maxillary showing the lateral alveolar bone needed to be removed before the extraction of the central incisor. e, f, g, h. Frontal aspect of the rabbit skull showing the central incisor and the supporting alveolar bone.

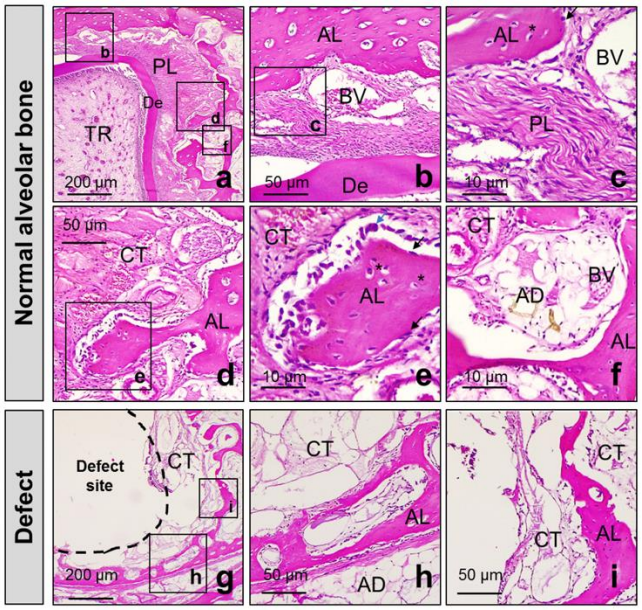
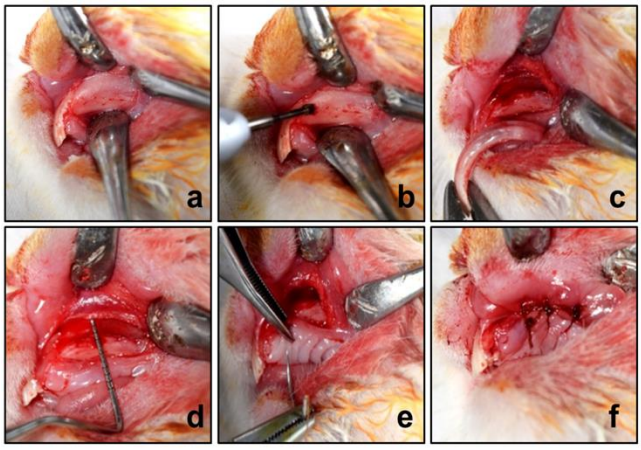


Figure 2. Histological characteristics of normal and defect model of alveolar bone
a-f. Normal alveolar bone. g-i. alveolar bone defect. AL, alveolar bone; PL, periodontal ligament; TR, tooth root; De, root dentin; BV, blood vessels; CT, connective tissue; AD, adipose tissue; black arrow, osteoblast; blue arrow, osteoclast; asterisk, osteocyte.

Histological analysis of a normal tooth and surrounding tissues revealed various different structures such as alveolar bone, periodontal ligament, blood vessels, connective tissues, adipose tissues, etc. There were a plenty of osteoblast, osteocyte at the alveolar bone site. These structures played an important role in nurturing the tooth and supporting soft and hard tissue (Figure 2a-f). In fact, the histomorphometric image of socket after extraction showed the lack of blood vessels, periodontal ligaments, the decreasing number of connective tissues, osteoblasts and osteocytes, which were the crucial part for bone formation (Figure 2g-i).



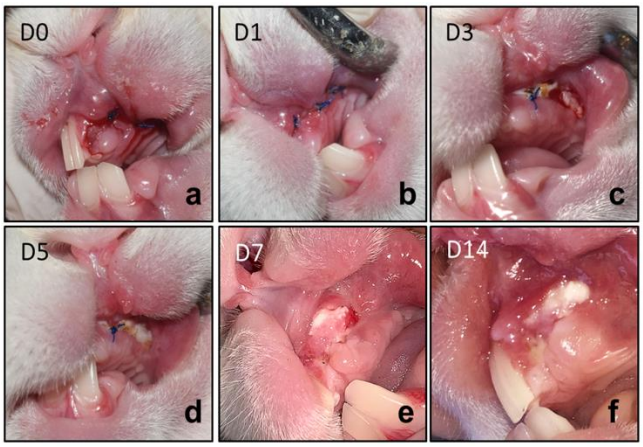
Supplement Figure 1. Alveolar bone defect model creation surgery
a. A linear incision around the marginal gingiva, extending to the distal-buccal aspect of the central incisor, along the curvature of the tooth is performed to expose the maxillary alveolus of the central incisor. **b.** A 2-mm round carbide bur is used to remove the lateral alveolar bone and expose the root. **c.** The root is gently luxated to the weakened lateral wall by an elevator and taken out by a forcep. **d.** The 2-mm alveolar defect is evaluated by a periodontal probe. **e.** Oral mucosa is sutured carefully with 5-0 resorbable Vicryl suture. **f.** The packed alveolar defect.

The surgical procedure were well-tolerated by all the rabbits (Supplement Figure 1). They were put under anesthesia using Ketamin without endotracheal intubation. In a few cases,

more Ketamin injection were required. The operation lasted approximately from 15 to 30 minutes and the management of bleeding went well during and after the surgery. The central incisor was easily removed after taking out the lateral alveolar ridge. No side effects such as loss of motion, convulsion, or respiratory disorder were reported. Postoperatively, all rabbits recovered well and maintained good health. The rabbits behaved normally, started eating at the first day after surgery, and maintained standard weight throughout the postoperative observation period. All of them survived until the day of sacrifice.

Wound healing process

On the clinical examination, in the first and second day the surgical site was clean and all the sutures remained intact. Swelling caused by the operation disappeared after 3 days and no bleeding were observed. However, from day 3, some rabbits felt more irritated and itchy at the wound so some of the sutures were broken due to the bites. Little bleeding was observed at the wounds; however it stops after several minutes, enhancing the soft tissue to heal continuously. There were no signs of inflammation or bleeding after day 5. The soft tissue healed completely after 2 weeks. (Supplement Figure 2)



Supplement Figure 2. Wound healing at the surgery site
a. Right after the surgery. **b,c.** 1 and 2 days after the surgery, the suture remains intact. **d,e.** 3 and 4 days after the surgery, some of the suture is broken due to the masticatory strength, cause bleeding at the surgical site. **f.** 5 days after surgery, bleeding stops. **g.** 2 weeks after, the wound is totally healed.

Osteological healing process

In representative 2-D radiographic images, a remarkable increase of contrast density was observed after 2, 4, 6, and 8 weeks compared to the normal and 0 week group. In the 0 week group, a C-shaped radiolucent corresponded to the extraction socket in clinical examination. After a period of 2,4,6,8 week, the radiolucent region was replaced gradually by an increase contrast density (Figure 3a-f).

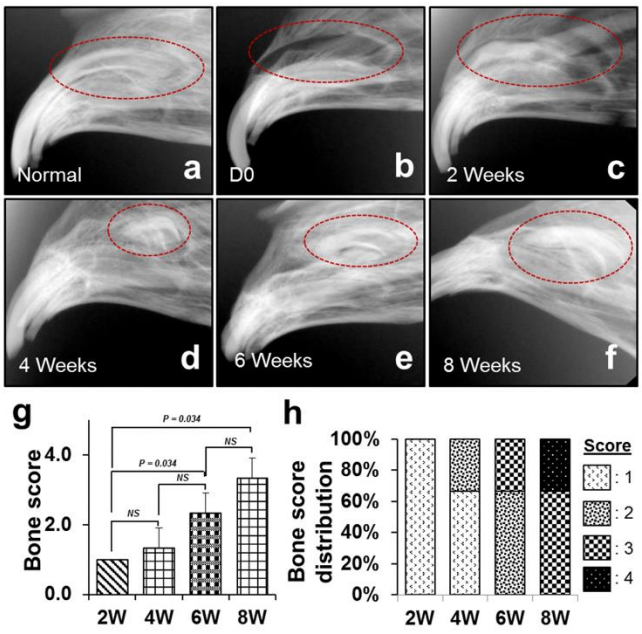


Figure 3. Alveolar bone healing on defect model examined by radiograph
a. Intact central incisor and supporting alveolar at a rabbit with no alveolar defect creation. b. At the time the alveolar defect created. **c, d, e, f.** Healing of osteotomy after 2,4,6,8 weeks of surgery. Note significant healing in bony tissue gradually along the time, mostly cortical bone. **g.** Histograms showing mean of newly formed bone scores after 2,4,6,8 weeks of surgery. **h.** Histograms showing the frequency distribution of newly formed bone scores after 2,4,6,8 weeks of surgery. NS: not significant.

These findings were in accordance with the score of newly formed bone which was measured according to Miloro et al. (Figure 3g). There was an upward trend in new bone formation starting from 2-week to 8-week, in which the percentage of newly formed bone in 8-week was as nearly as twice as in 2-week. A significant difference was found in the newly formed bone of 8-week and 6-week compared to

2-week (p<0,05). Looking deeper into the data in the bone score distribution chart, there was no newly formed bone detected at 2-week. 33,3% of rabbits scored 2, which were found to have new bone formation but the contrast was lower than the adjacent contiguous cancellous bone at 4-week. The rabbits scored 2 rose to 66,7% and the rest scored 3, which had the contrast higher than adjacent contiguous cancellous but lower than the cortical bone at 6-week. At 8-week, 66,7% of rabbits scored 3, exclusively there was the appearance of score 4 with 33,3%, which means 33,3% rabbits had the contrast higher than cortical bone (Figure 3h).

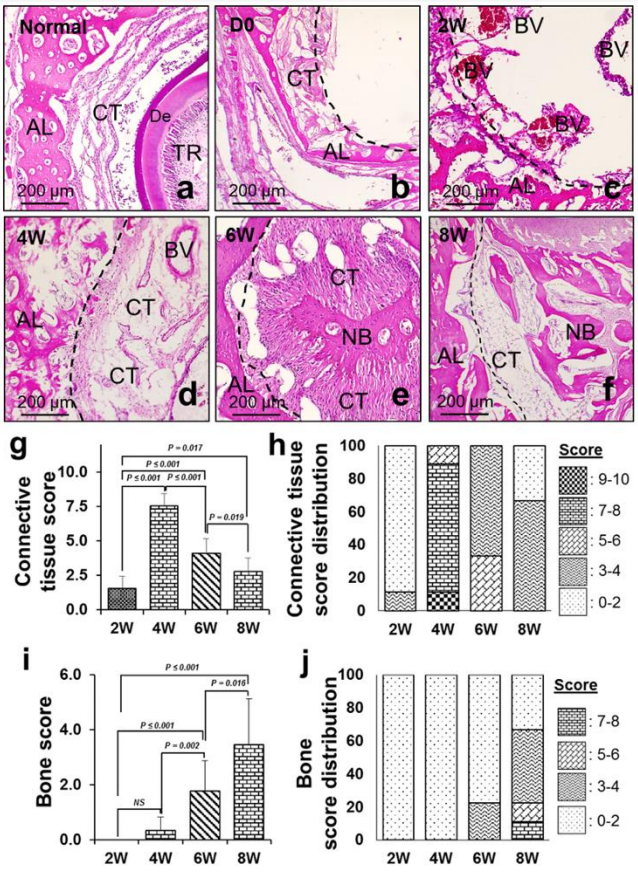


Figure 4. Histological aspects of bone and connective tissue regeneration on alveolar defect model.

a. Normal alveolar bone. **b.** alveolar bone defect at day 0. **c-f.** Tissue healing after 2,4,6,8 weeks of surgery g-h. Histograms showing mean and the frequency distribution of newly formed connective tisue scores after 2,4,6,8 weeks of surgery. i-j. Histograms showing mean and the frequency distribution of newly formed bone scores after 2,4,6,8 weeks of surgery AL, alveolar bone; TR, tooth root; De, root dentin; BV, blood vessels; CT, connective tissue; NB, new-formed bone. NS: not significant.

The comparison of histological images at the defect sites after 2,4,6,8 weeks showed bone healing pattern which correlated well with the radiographic findings. After 2 weeks, the blood vessels started to penetrate inside the defects. 4 weeks after the surgery witnessed the appearance and proliferation of connective tissues. The point of time after 6 weeks marks the presentation of new bone forming inside the connective tissues and the gradual decrease of connective tissue. Finally, the bone formed were mostly filled in the defects with the sink of connective tissue after 8 weeks. (Figure 4a-f).

Newly formed bone and connective tissue were scored based on the study of Han et al. These scores were in correspondence with the histological images, in which the new bone formation had an upturn from 2-week to 8-week, while the connective tissue rose from 2-week to 4-week then gradually dropped from 6-week. The difference of the bone score was significant at each point of time, except for the difference between 4-week and 2-week (p<0,05). A significant difference of connective tissue score was found at each point of time (p<0,05). (Figure 4g, i). Looking closer to the distribution of bone score which included 4 ranges of score: 0-2, 3-4, 5-6 and 7-8. All rabbits were scored 0-2 at 2-week and 4-week, which means less than 20% area of new bone formation were found. 6-week marked the appearance of 20% rabbits with score 3-4, which had 30-40% area of newly bone formed. 8-week witnessed the presence of 10% rabbits scored 5-6 and 10% of rabbits scored 7-8, the rabbits scored 3-4 were doubled compared to 6-week and 20% left belonged to rabbits with the score 0-2. The distribution of connective tissue contained 5 ranges of score: 0-2, 3-4, 5-6, 7-8 and 9-10. 80% of rabbits scored 0-2 and the rest scored 3-4 at 2-week. The majority of rabbits scored 7-8 and the remaining scored 3-4 and 5-6 with the same percentage at 4-week. At 6-week the score was distributed at 3-4 and 5-6 with the percentage of 60% and 40% respectively. 8-week marked the return of the score 0-2 and 3-4 with the rate of 40% and 60% respectively (Figure 4h,j).

Discussion

Alveolar bone in maxillary bone supporting tooth structure can be destroyed by various reasons including trauma, congenital deformity, tumors and cysts, infection and tooth loss. Therefore, such bone loss requires great effort in regeneration in order to conquer the appropriate volume of bone for dental implantation [17] [18]. Numerous approaches in tissue-engineering biomaterials have been made to augment and regenerate the volume of bone loss [19]. Alveolar defect model in animals have been developed recently to satisfy the need of application of novel biomaterials and evaluation of the proliferation of bone tissue [20] [21] [22]. The aim of bone grafting is to secure the volume and quality of bone needed for dental implantation or maintain the esthetic, anatomical and functional characteristics of the tooth and supporting structure. Therefore, we are developing an animal model with alveolar defect, imitating the three-dimensional morphology of a patient suffering alveolar and palate cleft, periodontitis, tumors and cysts, which leads to tooth extraction and bone loss afterwards.

Different types of animal models have been proposed to test the efficacy of bone-substitute biomaterials such as rats, rabbits, dogs, swine, goats, sheep, primates [23], [24], [25], [26], [27]. Even though there are several methods of creating alveolar defects such as transgenic, teratogenic cleft models, surgical created defects proved to be more suitable because they could standardize the size of defect and prevent the variability of the cleft in genetic-related models [28]. First surgical-created defect was reported by Harvold in 1950, which demonstrated a 2-mm alveolar and palatal defect in rhesus monkeys [29]. Compared to small rodents, bigger animals such as monkeys and dogs appear to have more similar skeletal macrostructures to humans, therefore various researches have been conducted to introduce different surgical procedures and their modification, mostly on dogs and monkeys [30]. However, the high cost of housing, operation and the amount of molecular biomaterials available requires the findings of a smaller animals for creating an alveolar defect which is also acceptable and meets the need of cost effectiveness but still remains the optimal result [28]. In our study, rabbits were chosen since they are easy for husbandry and observation, have a faster vital ability of gestation and maturity. Furthermore, bone mineral density and fracture between human and rabbits have several things in common. When compared to other rodents, rabbits demonstrate a quicker skeletal metabolism and bone regeneration rate [14] [26].

We were able to create an alveolar defect in the maxilla front region, along with a nearby central incisor which imitates the clinical case of a patient suffering from alveolar bone loss after periodontitis, trauma, tumors and cysts, congenital cleft, etc. We follow the method of extracting the central incisor by removing the lateral bone introduced by Maslamani et al. [31]. Because of the extraordinary C-curved shape of the central incisor, it is impossible to extract the tooth in a normal way. Indeed, it requires some lateral bone removal and luxation afterwards. The surgery was carried out in 15-30 minutes while the rabbits were put under anesthesia with Kentamin. There were no signs of inflammation or bleeding after day 5. The soft tissue healed completely after 2 weeks. Radiographic results presented in this article showed a significant increase in ossification surrounding the alveolar defect, mostly cortical bone. The volume of mineralized tissues rises gradually after 2, 4, 6, and 8 weeks. Histologic analysis demonstrated a process of bone remodeling from the appearance of blood vessels inside the defect to the proliferation of connective tissues, leading to the initial bone forming at 6 weeks and ending up with connective tissues and bone filled in the defect. This pattern corroborates our radiographic findings, suggesting the time needed for alveolar bone regeneration in a rabbit model.

There have been a lot of methods described the procedure of creation an alveolar defect in small-sized animals [32] [33] [18]. An alveolar defect model reported by Xu et al. was performed on rats by extracting a molar tooth and applying bone wax. [34]. They were successful in controlling the bone regeneration process; however, the anatomical structure was not corresponded with the clinical alveolar defect in maxillary region caused by congenital, trauma reasons. Kamal et al. described an alveolar defect in rabbit by extracting the central incisor and established a communication between oral and nasal cavity. This model was closer to imitate a clinical case of a patient suffering from alveolar cleft and palate [35]. However, the aim of our model was to create

a model for mimicking the cases of the patients who have injury, periodontitis, tumors or cysts in maxilla that results in the extraction of current tooth and ends up with remarkable bone loss. This animal model helped us to test the efficacy of the tissue-engineering bone substitute materials in a non-true healing defect and reduce the amount of time needed for complete bone regeneration, which is a critical element for stability of implant afterwards. Takano-Yamamoto et al., Sawada et al., Puumanen et al., Pilanci et al. and Kim et al. also described an alveolar defect model with immediate grafting. [36], [37], [18], [38]. The disadvantage in our study was the lack of micro-CT which provides a three-dimensional overview of the alveolar defect and bone regenerated.

Conclusions

To sum up, our study reveals that it is possible to perform a sizable alveolar defect in rabbits. The operative site has a similar size and is in the same region of human, this procedure doesn't need any magnifying apparatus or micro instruments.

Acknowledgments

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Declaration of Interest

The authors report no conflict of interest.

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