Animal Study of tooth extraction wound healing using collagen sponge matrix “TRE-641”.
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1. Introduction

Tooth extraction is one of the most routinely performed treatments among dental surgical techniques, but essentially it is a surgical treatment that cannot avoid producing the wound on the living body\(^1\). In particular, tooth extraction wound which is produced after difficult tooth extraction with severely injured alveolar bone, or large tooth extraction wound accompanying the "bone defect" in the cortex of the alveolar bone that may develop healing failure such as dry socket do not always take satisfactory healing process by open treatment therapy that is performed through ordinary tooth extraction. In some cases, ostitis develops concurrently and its injury reaches the bone\(^1\). Furthermore, in patients with other diseases (patients with blood disease, patients receiving dialysis, patients treated by drug therapy such as coagulant and steroid drug, patients with liver disease, patients with hemorrhagic disease such as diabetes mellitus, patients with infectious property, patient with difficulty in bite hemostasis due to physical or mental disorder), there is a possibility of aggravation of systemic condition or of worse prognosis due to uncontrollable bleeding after tooth extraction\(^1\).

Thus, authors et al. have provided easy treatment method for the above mentioned patients and developed atelo-collagen tooth extraction protective materials\(^2,3\) that do not prohibit healing promotion with good results in clinical trial\(^4\). This is the product (Teruplug\(^\circledR\) TRE-641, hereinafter referred to as “TRE-641”) processed into the bullet shape using as the design base the mixed collagen sponge composed of fibrillar atelo-collagen (FAC) and heat-denatured atelo-collagen (HAC), which is the defect filling portion of artificial dermis (Terudermis\(^\circledR\)) that was effective for filling the dermis defect in the dermal region\(^5\) or filling the mucous defect in the buccal region\(^6\).

In this study, tissue affinity and wound healing of TRE-641 in the tooth extraction wound that were experimentally produced on the cutting tooth and the premolar of the beagle dogs were histopathologically examined using open treatment, conventional general
treatment or commercial collagen hemostat as controls. In particular, with the premolar, the alveolar bone was scraped after tooth extraction, thus treatment effect of TRE-641 on bone cortex defect wound are also discussed.

2. Materials and experiment methods

2.1 Production of TRE-641

TRE-641 was produced according to the production method\(^7\) of collagen sponge of Terdermis\(^8\). As material for collagen, atelo-collagen\(^8\) (Koken Co., Ltd.), which is known as low antigenic was used.

Powder atelo-collagen was dissolved into acid solution, a part of it was neutralized and refibrization was made (production of fibrillar atelo-collagen), a part was heat denatured in the solution state (production of heat denatured atelo-collagen). Fibrillar atelo-collagen and heat denatured atelo-collagen was mixed with a ratio of 9:1, and finally its concentration was regulated to 25 mg/ml. This solution was infused separately into a bullet type container, freeze-dried and modeled into a sponge state. Sponge put in the container was heated at 110 °C for 2-3 hours within the vacuum drier, and after crosslinking (dehydrothermal crosslink) was introduced, TRE-641 was obtained (Photo 1).

2.2 Experimental animals

Six male beagle dogs (Nalc Production, Nosan Corporation) weighing 8.3-11.8 kg were used.

As feedstuff, powdered feed for dog CD-5 (Clea Japan, Inc.) was given 300g once per day from preparatory rearing period through experimental period.
2.3 Experiment methods

Beagle dogs were preadministered with epinephrine physiological saline (Bosmin® Daiichi Seiyaku Co., Ltd.: diluted to 30,000 fold with physiological saline) under anesthetic of pentobarbital (Nembutal® Dainippon Pharmaceutical Co., Ltd., 25 mg/kg i.v.) with an aim of hemostasis to the site in the gingiva to be extracted, then 2 kinds of small tooth extraction wound and large tooth extraction wound shown in Fig. 1 were made at 4 sites per one dog, totaling 8 sites.
[1] Small tooth extraction wound group

Maxillary bilateral second incisor (J₂) and mandibular bilateral third incisor (J₃) were dislocated using a dental elevator and extracted applying rotation movement using forceps. Then, it was fully cleaned and bleeding was arrested using epinephrine physiological saline (diluted to 30,000 fold) and autoclave sterilized paper wiper (Kimwiper®, CRECIA corporation) to make a small tooth extraction wound, to which the following treatment was performed.

Small type TRE-641 was cut into 20 mm in length (weight at that time, 29.0 mg), and inserted into the tooth extraction wound, putting pressure. As a control, the same amount of absorbable hemostat (Though there are multiple kinds, sponge type of atelo-collagen which is reported to be low antigenic was selected, hereinafter referred to as collagen hemostat) was weighed out and similarly inserted. As another control, open wound group was made, in which treatment has not been done after tooth extraction and arrest of bleeding. In all these 3 groups, suture of mocosa was not carried out.

[2] Large tooth extraction wound group

Mandibular bilateral third premolar (P₃) and mandibular bilateral fourth premolar (P₄) are composed 2 dental roots, respectively, thereby at first, each premolar and surrounding gingival mocosa were peeled off, and a cut was given into the premolar using a dental drill (disc), and separated into two parts from the base. Then tooth extraction was done in the similar way to small tooth extraction wound. Furthermore, the alveolar bone at the boundary of 2 tooth roots was sharpened to arrange the shape, pouring physiological saline, using a dental drill (bar) with the tip diameter of 3 mm so as that all the large tooth extraction wounds will become almost the same size (8 mm×3 mm×7 mm in depth), thereby, large tooth extraction wound (surface defect of the alveolar bone) was made (Photo 2). The produced tooth extraction wound was fully cleaned and after arrest of bleeding, the following treatment was carried out, in the similar way of small tooth extraction wound.

In the TRE-641 group, large type of TRE-641 (weight, 56.7 mg), and collagen hemostat that was weighed out as the same weight was inserted, putting pressure on the
tooth extraction wound, respectively. It was expected that the aperture may be large as it is, and infiltration and retention of feedstuff residue, dropout of subject specimen, thus, ligature suture of mucosa was performed with 5.0 nylon suture. At the open wound, nothing was inserted into the tooth extraction wound and ligature suture of mucosa alone was carried out.

2.4 Infection measure

After treatment, ampicillin (Perlecin®, Fujisawa Pharmaceutical Co., Ltd.) was administered intramuscularly (20 mg/kg) once.

Furthermore, fosfomycin antibiotics (Fosmicin dry syrup®, Meiji Seika Kaisha, Ltd.) was administered with powdered feedstuff for 5 days after tooth extraction (100 mg/kg/day).

2.5 Macroscopic observation and care

Macroscopic observation of the wound was performed at 4 and 7 days after treatment and since then, every week until the time of autopsy under general anesthetic of pentobarbital. And at the time, in the large tooth extraction wound, if it is become clear that thread gets out of place, resuture was performed with 5.0 nylon suture thread, while if it is judged that gaping was created due to membrane necrosis, resuture was not performed particularly.
2.6 Autopsy

At 1, 2, and 4 weeks after treatment, 2 animals were sacrificed each by bleeding under general anesthetic of pentobarbital, and presented for autopsy (At any autopsy period, small tooth extraction wound and large tooth extraction wound was arranged so as that n=3 for the TRE-641 group and collagen hemostat group while n=2 for the open wound group). Extirpation was done including sample application site using a cast cutter and fixed in the 10 % neutral buffering formalin.

2.7 Histopathological examination

Material was fixed with 10 % formalin (more than 1 week), and decalcification was carried out at room temperature for 3 - 4 weeks using 5 % formalin formate. Then it was sectioned in the direction that existing alveolar bone become U shape on section, and paraffin section was made according to the usual manner, and stained with H&E and toluidine blue, and histopathologically observed.

3. Results.

3.1 Macroscopic observation

[1] Small tooth extraction wound

At the time of treatment, TRE-641 absorbed rapidly the blood which effused from the tooth extraction wound surface after arrest of bleeding and turned to red. Collagen hemostat available on the market had tendency to repel effusion, while it clings and adheres to hematoma created on the inner wall of tooth extraction wound which provides some difficulties in inserting. In the open wound, it took more time until arrest of bleeding when compared to TRE-641 or collagen hemostat treatment groups.

At 4 days after treatment, wound closure has not finished, and the state of each subject specimen was macroscopically confirmed. In TRE-641, red color tinge has already disappeared, and almost all were white through pink tinge, and a part had transparency. And depression feeling was not observed. Almost all the collagen hemostat had turned red, some were red through black, or brown in color, and in 6 of 9 patients, depression feeling was observed. Open wound was filled with hematoma or
fibrin, and in 2 of 6 patients, depression feeling was observed.

Then, in all the patients, almost all the wound was closed due to wound contraction and regeneration of mucosal epithelium since 2 weeks after treatment.

[2] Large tooth extraction wound

With regard to operability and hemostasis, the same results were obtained as those in small tooth extraction wound. At the time of mucosa suture, there were no problematic cases.

At 4 days after treatment, in 1 of 9 patients in the collagen hemostat treatment group, gaping was observed. And collagen hemostat which turned gelatin state was macroscopically observed. Except this, until at 4 weeks after treatment, particular marked change was not observed macroscopically.

3.2 Histopathological findings

[1] Small tooth extraction wound

In all the 3 patients of TRE-641 group, residue rich in collagen fiber bundle which may be derived from TRE-641 was observed within the extracted tooth wound alveolus at 1 week after treatment. And in the space between collagen fiber bundles, infiltration of neutrophil, histiocyte, and fibroblast were observed. Even at 2 weeks after treatment, rich collagen remained, and in the space of collagen fiber bundle which folds well, tissues (hereinafter newly generated soft tissue which may be derived from TRE-641 is called dermis-like tissue7) where the capillary or fibroblast of recipient infiltrated in a scattering manner were observed. And at the marginal side of tooth extraction wound which is close to the existing alveolar bone, osteoblast was observed. And in 1 of 3 patients, regeneration of woven bone was started. At 4 weeks after treatment, in all the 3 patients, in the whole area within tooth extraction wound, construction of woven bone was observed. At the surrounding margin of tooth extraction wound, there was already no dermis-like tissue in part, and they were replaced into the loose connective tissues which are rich in woven bone and vessels. And at the space of woven bone in the central portion of tooth extraction wound, in part, residue of dermis-like tissue was observed.
The height of surrounding alveolar bone was high enough, thus alveolar bone absorption may be not so strong during observation period (Photo 3A).

Collagen hemostat group at 1 week after treatment, residue of collagen hemostat was at only the margin or bottom of tooth extraction wound, and its amount was decreased when compared with TRE-641. Within collagen hemostat, only a few neutrophil invaded, and at the surrounding of collagen hemostat, neutrophil, histiocyte, and fibroblast were observed. At 2 weeks after treatment, collagen hemostat had already disappeared, and in 2 of 3 patients, at the bottom and margin of tooth extraction wound, regeneration of osteoblast and woven bone were observed. At 4 weeks after treatment, in all the 3 patients, woven bone was observed in portion surrounded by the alveolar bone, however, in 1 of 3 patients, height of surrounding alveolar bone was decreased (Photo 3B).

In the open wound group, at 1 week after treatment, in both 2 patients, the whole tooth extraction wound was filled with hematoma. And at 2 weeks after treatment, one was filled with hematoma, while another was filled with blast granulation tissue. In both patients, regeneration of osteoblast and woven bone was observed. At 4 weeks after treatment, in all 2 patients, the whole tooth extraction wound was filled with woven bone (Photo 3C).
[2] Large tooth extraction wound

In all the 3 patients of TRE-641 group, at 1 week after treatment, collagen remained in rich, and infiltrations of neutrophil, histiocyte, and fibroblast were observed from the surrounding (Photo 4A). At 2 weeks after treatment, in all the 3 patients, histiocyte, fibroblast and capillary invaded into the collagen, and turned into the good dermis-like tissue (Photo 4B). And 2 of 3 patients, at the bottom of the tooth extraction wound, start of regeneration of osteoblast and woven bone was observed. At 4 weeks after treatment, in all 3 patients, construction of woven bone was observed in the whole area of tooth extraction wound (Photo 7A). At the surrounding of tooth extraction wound, dermis-like
tissue was already disappeared, and replaced by loose connective tissue which is rich in woven bone and vessel. It was observed that at the center portion of tooth extraction wound, in part, dermis-like tissues remained, and they replaced smoothly into woven bone (Photo 4C, D). And bone absorption by osteoclast in the existing alveolar bone was observed only in 1 patients at 2 weeks after treatment, out of 9 patients at 1, 2, 4 weeks autopsy. And in other individual, absorption and deformation of the existing alveolar bone was not observed (Keeping U shape, Photo 7A).

In the collagen hemostat group, at 1 week after treatment, collagen hemostat remained in the tooth extraction wound surrounded by the alveolar bone. Infiltration of neutrophil was only found at the surrounding portion. And in all the 3 patients, upper end of the alveolar bone, prosperous bone absorption by osteoclast was observed (Photo 5A). At 2 weeks after treatment, in part, collagen hemostat residue was observed,
however, almost all became blast granulation like tissues mainly composed of fibroblast, and at the bottom of tooth extraction alveolus, regeneration of osteoblast and woven bone was observed (Photo 5B). Furthermore, in all the 3 patients, active bone absorption of the existing alveolar bone due to osteoclast as well as reformation (U shape into J shape, Photo 5B) of the alveolar bone were observed. At 4 weeks after treatment, in 1 of 3 patients, collagen hemostat was found to remain as it is with scarce cellular infiltration. In the individual patient, neither regeneration of woven bone nor wound healing reaction after tooth extraction, that is, formation of granulation tissue within tooth extraction wound, was observed. In the remaining 2 out of 3 patients, collagen hemostat already did not remain and in the whole area of tooth extraction wound surrounded by the alveolar bone was filled with regenerated woven bone. At the same time, at the upper end of existing alveolar bone, height of the alveolar bone was decreased due to active bone absorption by osteoclast, resulting in that the alveolar bone was deformed into not U shape but J shape or shallow cup type (Photo 7B).
With regard to open wound, at 1 week after being left alone, the whole tooth extraction wound alveolus was filled with hematoma, while bone absorption is not so markedly observed (Photo 6A). At 2 weeks after treatment, in both 2 patients, tooth extraction wound alveolus was filled with blast granulation like tissues and hematoma. Furthermore, good regeneration of woven bone was observed at the bottom and periphery. However, at the same time, at the upper end of existing alveolar bone, prosperous existing
alveolar bone absorption by osteoclast as well as reformation of bone (U shape into J shape) was observed (Photo 6B). At 4 weeks after treatment, the whole tooth extraction wound was filled with woven bone. And absorption of existing alveolar bone by osteoclast resulted in a decrease in height or reformation of the alveolar bone in 2 patients (Photo 7C).

Based on above, it was suggested that at large tooth extraction wound, the amount of regenerated woven bone, at 4 weeks after treatment, in TRE-641 in which a decrease in height of the alveolar bone was not observed surpassed histologically the other group.
4. **Discussion**

In this experiment, effects of atelo-collagen protective material (TRE-641) that was developed for use of the tooth extraction wound were evaluated in large and small tooth extraction wounds of beagle dogs, comparing with open wound or commercial collagen hemostat.

At first, the situation controlling infections that is the premise of experiments is described. As is described in sections of experimental method and infection measures, control was done by administration of antibiotics (orally and intramuscularly), and large tooth extraction wound were sutured using mucosal ligature. As a result, not only in tooth extraction wound group, but in small tooth extraction group where the collagen sponge becomes in the uncovered state, symptoms of localized bacterial infection were not observed. Terudermis® that is the design base of this material has achieved clinically satisfactory results in the area of plastic surgery or oral surgery, but in any area, there have been no reports describing that collagen sponge of Terudermis® became infection source. Based on the above, it is predicted that there will be no anxiety in the clinical tooth extract wound. There were no infections observed in control, collagen hemostat group.

The following phenomena were observed in both large and small tooth extraction wounds in this experiment: TRE-641 showed a good absorption of initial blood and
exudate, whereas, commercial collagen hemostat did not retain hematoma and hematoma clung onto the surface of hemostat. For the open wound group with difficulty in arresting bleeding, both TRE-641 group and collagen hemostat group showed good initial hemostatic actions, however hemostatic mechanism of both were not perfectly same. TRE-641 may have physical action such as increased area contacting the air in the collagen and collagen sponge by holding hematoma efficiently other than biochemical hemostat on the surface of collagen. In small tooth extraction wound, the color of collagen hemostat turned into red to brown at 4 days after treatment, the reason may be that hematoma that could not invade into the hemostat retained around the hemostat. It is expected that this phenomenon may affect subsequent histological change after that.

With regard to histological change in collagen hemostat, absorption was observed in early stage over 1 through 2 weeks in the small tooth extraction wound, while phagocytosis and absorption were found (except for one case) with time in rather later stage in the large tooth extraction wound. Whereas, in collagen sponge of TRE-641, such an absorption in early stage was not observed. At 1 week after treatment, good invasions of histiocyte, fibroblast, and blood capillary were observed, which suggested strong promotion of localized wound healing. At 2 weeks after that, large and small wounds were changed into the dermal like tissue, at 4 weeks after, specific tissue aspect was observed where residual dermal like tissue in the center continued smoothly to the normal woven bone. Although woven bone regeneration at 2 weeks after was observed in only 2 of 3 cases at 4 weeks after treatment, almost all the regions of the tooth extraction tooth extraction cavity turned into the woven bone in all the patients, which bore comparison with open treatment or collagen hemostat. Based on these observational results, it was considered that TRE-641 changed into dermal like tissue at the initial stage, then mineral deposition occurred with this tissue as matrix, leading to the woven bone. This suggests a possibility that collagen material of TRE-641 (FAC-HAC compound) accelerates the bone formation.

Lastly, bone defect repair process is mentioned. Among tooth extraction wounds that were adopted this time, large tooth extraction wound, tooth extraction wound of the premolar, is not "tooth extraction wound" in the strict sense of the word, rather it is "bone defect" because a part of the alveolar bone was scraped by drilling. So, its wound repair process is different from that of tooth extraction wound for which tooth extraction was done carefully so as not to injure the alveolar bone and its bone absorption of the alveolar
bone may be increased. However, recently the report has been published\textsuperscript{10} describing bone induction ability of recombinant BMP using the bone defect wound after tooth extraction at P\textsubscript{3} and P\textsubscript{4} of the beagle dogs as evaluation site. This tooth extraction wound is considered to be generalized as evaluation system. In fact, in extraction of the impacted tooth, which is difficult extraction, the healthy alveolar bone has to be injured to some extent, which results in the wound that should be termed as "bone defect" rather than "extracted wound." This may subsequently lead to difficulty in arresting bleeding or dry socket, furthermore, it may develop disease such as buccal stoma of the maxillary sinus (perforation). As is suggested by this treatment, all of these may be good application for TRE-641 and its effect can be expected.

Characteristics of each specimen that was noted in small tooth extraction wounds were more clearly observed in large tooth extraction. That is, absorption images of residual alveolar bone and its resultant alveolar deformation were observed in a total of 8 of 9 patients of autopsy at 1, 2, and 4 weeks after in collagen hemostat, control. In the remaining 1 patient, bone absorption was not observed, but cellular invasion into the wound as well as progression of healing was not found. The similar bone absorption or alveolar bone deformation were also observed in the open wounds, although not so significant as in collagen hemostat, and existing alveolar bone that was originally U-shaped was deformed due to bone absorption into J-shape or shallow-cup (a total of 4 of 4 patients of autopsy at 2 and 4 weeks.). While, in TRE-641, deformation of the alveolar bone was observed in only 1 of 9 patient out of total of autopsy at 1, 2, and 4 weeks after. Based on the above, it is concluded that if TRE-641 is applied to the tooth extraction wound, maintenance of residual alveolar bone is expected. In general, in the normal bone tissue, the coupling of bone absorption and bone formation that osteoblast repairs the portion which was broken and absorbed by osteoclast is always performed\textsuperscript{11}. However, if mechanical stimuli are applied such as the changes in bite pressure, osteoclastis is induced around it and bone absorption progresses rapidly\textsuperscript{12}. In this experiment, deformation of the alveolar bone due to bone absorption was observed in the open wound, thus, such a bone absorption may happen and cannot avoid in the tooth extraction wound that has a possibility to give injury to the alveolar bone like extraction of impacted tooth whose extraction is very difficult. Whereas, TRE-641 suggested a possibility of acting as protective material of the alveolar bone that received injury. The above is summarized in the schematic diagram (Fig.2).

With harmful effects in the case of deformation of the alveolar bone due to bone
absorption, the influence of deformation on other dentitions is expected. Recently, although the treatment using an artificial tooth root (implant) has been increased, it is important that artificial tooth root is closely connected with the alveolar bone tissue around after implantation and stabilized in the earlier stage\textsuperscript{13).} It is clear that if the bone at the implanted site is deformed or its height is decreased, the area where the artificial bone root has contact with the bone will be decreased and it will be difficult to obtain the stability. So, it was suggested that applying TRE-641 to the tooth extraction wound in advance may provide a possibility to prevent the alveolar deformation at the site to which implant applied.

5. Conclusion

Two kinds of tooth extraction wound, large and small, were made in the beagle dog, and tooth extraction protective material TRE-641 was inserted. Then it was compared with open treatment or commercial atelo-collagen absorptive local hemostat with time. As a result, in the small tooth extraction wound, hemostat effect and woven bone regeneration of TRE-641 were observed. Furthermore, in large tooth extraction wound, in addition to that, deformation of the alveolar bone was not observed, which ensures the good point to protect the alveolar bone after tooth extraction. In other control group, height was decreased by the residual alveolar bone absorption, therefore, in TRE-641 group at 4 weeks after, when the alveolar bone was preserved, it was histopathologically suggested that woven bone regeneration amount of the tooth extraction tooth extraction cavity exceeds that in other control group. A possibility of mineral deposition of TRE-641 that turned into the dermal like tissue was suggested as mechanism of turning to woven bone in TRE-641.

Based on the above, clinical usefulness of TRE-641 can be expected for the tooth extraction wound that is difficult to remove the tooth, showing severe injury of the alveolar bone (subsequent alveolar bone absorption is expected) or tooth extraction wound that has a possibility of treatment failure after tooth extraction.