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The effects of Hydroxyapatite/Chitosan (H/C) Block scaffold in One-wall Intrabony Defect of Beagle Dogs

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The effect of Hydroxyapatite/Chitosan (H/C) block scaffold and HA/β-TCP (H/T) particle on the regeneration was evaluated in one-wall intrabony defects in beagle dogs. Six male beagle dogs were used in this study. H/C block scaffold was manufactured by freeze-dried method. One-wall intrabony periodontal defects (4 × 4 mm) were surgically created. Prepared defects were randomly assigned and treated as follows. In surgical control group (C) the defects were not filled. In HA/β-TCP group (H/T), defects were filled with HA/β-TCP particle bone graft material. In HA/Chitosan block group (H/C), defects were filled with HA/Chitosan Block scaffold. In all groups, Mean values of regenerated bone, regenerated cementum, connective tissue, and epithelium were measured. H/C group showed less inflammatory reaction than other groups. It revealed good biocompatibility. This group also showed better cementum regeneration than any other group. However, due to the relatively rapid resorption of chitosan, space maintenance for regeneration seems to have been compromised. Bone regeneration was affected adversely with this rapid resorption. More favorable bone regeneration was found in H/T group because proper space was maintained with Β-TCP.

Key words: Periodontal regeneration, Hydroxyapatite/ Β-TCP (H/T), Hydroxyapatite/chitosan block bone

Introduction

The ultimate goal of periodontal therapy is the regeneration of the attachment apparatus. New cementum, new bone, and new periodontal ligament formation are required. Varieties of bone grafts, and membranes have been developed. Many clinical procedures have been tried for regeneration. Bone grafts were widely investigated for periodontal regeneration. Autograft, allogenic material, alloplastic material, and xenogenic material have been widely studied. New bone formation was accomplished with application of bone grafts in combination with GTR.[1)] Biocompatible materials such as hydroxyapatites, calcium phosphates and inorganic bone graft materials have been used alone or in combination with GTR. The various results of bone formation were found.[2)] Perfect regeneration could not be achieved with conventional techniques.[3,4)]

Tissue engineering strategies are recently applied for periodontal regeneration to overcome limitations of conventional regenerative techniques. For tissue engineering, scaffold provides a solid framework for cell growth and differentiation. It should have sufficient mechanical stability. Scaffolds must be resorbed slowly to maintain proper space. This space would be replaced by new bone. Scaffolds should be biocompatible without any inflammatory reaction. It has to be manufactured, sterilized, and handled easily.[5)] The scaffold should also have sufficient porosity to accommodate osteoblasts, periodontal ligament cell. It should support cell proliferation and differentiation enhancing bone tissue formation, cementum and new attachment. High interconnectivity between pores is also desirable for uniform cell seeding, and diffusion of nutrients.[6)]

Various scaffold materials have been used *in vitro*. Bone formation was reported in a study with culturing stromal osteoblasts in a three dimensional biodegradable poly (lactic-co-glycolic acid) foam.[7)]

Healing of injured connective tissue was enhanced by Chitosan.[8)] Chitin is a natural polymer of N-acetylglucosamine. It comprises a component of the exoskeleton of organisms such as shells and cuticles of arthropods.[9)] Chitosan has excellent potential as a structural base material for a variety of engi-

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neered tissue system.[10] It has been reported to enhance periodontal tissue regeneration.[10-12] However, several inherent disadvantages have been observed with chitosan as a scaffold material. It includes weak structural integrity, variable degradation rates. Poor physical property of Chitosan results in unfavorable outcomes.

Hydroxyapatite is widely investigated material for bone regeneration. Hydroxyapatite-chitosan block scaffold is manufactured by combination of chitosan and hydroxyapatite to enhance physical properties. Purpose of this study is to evaluate biocompatibility of H/C block scaffold and HA/β-TCP particle on the regeneration of one-wall intrabony defects in beagle dogs and to compare periodontal regeneration between block and particle graft.

Material and methods

Animals

A total of six male beagle dogs, each weighing about 15 kg, were used in this study. The animals had intact dentition and a healthy periodontium. Animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea. The animals were fed a soft diet throughout study, in order to reduce chances of mechanical interference with the healing process during food intake.

H/C block scaffold

H/C scaffold was manufactured by freeze-dried method. Chitosan solution was prepared by dissolving chitosan (2wt%) in 0.2 M acetic solution. H/C solution was made by dispersing hydroxyapatite (HA, Sigma-Aldrich co., USA) in the chitosan solution (Chitosan : hydroxyapatite = 7 : 3). H/C solution was poured in Φ 6 mm × 12 mm teflon mold. Above 5 hours, it was refrigerated in -70°C. It was freeze-dried under 6 mTorr by freezing dehydrator for more than 3 days. The residual acetic acid was neutralized by 1 M NaOH solution, and then washed with the distilled water (above 3 times) and it was soaked in bovine serum albumin. The solvent was completely dried by freeze dry for 3 days. H/C hybrid scaffold was manufactured. The grain size was 10^2 nm.

H/T particle bone graft

HA/ β TCP particle bone graft (Osteon®, Dentium. Republic of Korea) is a newly developed alloplastic material containing 70% HA and 30% β-TCP. The interconnected porous scaffold is comprised from biocompatible HA, while the surface is coated with bioresorbable β-TCP. Particle size is 0.5-1.0 mm (Kim et al., 2007).

Surgical procedures

Six male beagle dogs were used. 4 × 4 mm one-wall intrabony periodontal defects were surgically created bilaterally at the distal sides of the mandibular second premolars and mesial sides of the fourth premolars under general anesthesia with sterile conditions in an operating room using atropine 0.05 mg/kg SQ, xylazine (Rompuns, Bayer Korea, Seoul, Korea) 2 mg/kg, ketamine hydrochloride (Ketalars, Yuhan Co., Seoul,Korea), and 10 mg/kg IV. Dogs were placed on a heating pad, intubated, administered 2% enflurane, and monitored with an electrocardiogram. After disinfecting the surgical sites, 2% lidocane HCl with epinephrine 1 : 100,000 (Kwangmyung Pharm., Seoul, Korea) was administered by infiltration at the surgical sites. Prepared defects were randomly assigned an experimental condition and treated as follows. Flaps were sutured with 5-0 resorbable suture material (Polyglactin910, braided absorbable suture, Ethicon, Johnson & Johnson Int., Edinburgh, UK). On the day of surgery, the dogs received 10 mg/kg IV of the antibiotic cefazoline. The dogs were sacrificed at 8 weeks after the experimental surgery.

Experimental group

Surgical control Group (C)

Created defects receive nothing, flaps were repositioned.

H/T particle bone graft group (H/T)

Bone graft particles were hydrated with sterile saline and applied to defect.

H/C Block scaffold group (H/C)

Blocks were shaped with #15 scalpel and scissors to fit defect and applied to defect.

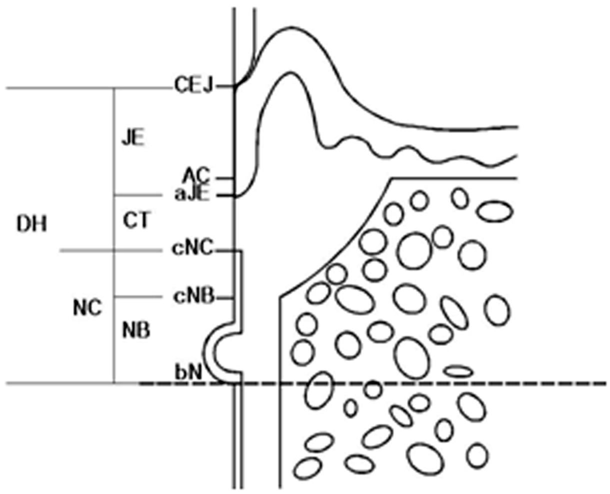
Evaluation method

Clinical Observation

Following surgical procedure, surgical sites were examined if there were any inflammatory reactions or uneventful healing.

Histologic analysis

Tissue blocks, which included teeth, bone, and tissue, were removed, rinsed in saline, then fixed in 10% buffered formalin for 10 days. After being rinsed in water, the block section were decalcified in 5% formic acid for 14 days, and embedded in paraffin. Serial sections, 5 μm thick, were prepared at intervals of 80 μm. The four most central sections from each block were stained with hematoxylin/eosin (H-E) and examined using a light microscope. The most central section from each block was selected to compare histologic findings between groups. Computer-assisted histometric measurements were obtained using an automated image analysis system (Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, U.S.A) coupled with a video camera on a light microscope (Olympus BX50, Olympus Optical Co., Tokyo, Japan). Sections were examined at 20x magnification.



DH : defect height JE : junctional epithelium migration
CT : connective tissue adhesion NC : new cementum regeneration
NB : new bone regeneration aJE : apical extent of junctional epithelium
bN : the base of the reference notch CT : connective tissue

Figure 1. A schematic diagram depicting the experimental design, the landmarks and the parameters used in histometric analysis.

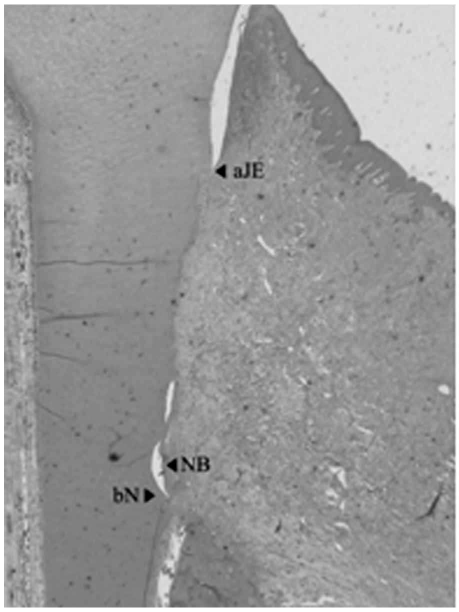


Figure 2. Section of surgical control group (× 20).

Histometric Analysis

For the histometric analysis, the cementoenamel junction (CEJ) and the notch were used as reference points. The alveolar crest (AC) point was gained by subtracting the distance between the AC and CEJ from the CEJ measured at the experiment. (Figure 1)

Results

Clinical observations

Surgical procedures were uneventful and without complication in all dogs. Wound closure was successfully maintained throughout the experiment for all defects. Healing process was uneventful.

Histologic findings

Surgical control group

The apical migration of junctional epithelium was greatest. In some slides, a little amount of new cementum and bone had formed in notch region along the root surface. There was little or no sign of inflammatory cell infiltration (Figure 2).

H/T particle bone graft group

The apical migration was greater than HA/chitosan block scaffold group, bone regeneration was greatest and the coronal portion of regenerated bone was relatively distant from root surface. Cementum regeneration was observed which was greater than surgical control group. There was little or no sign of inflammatory cell infiltration. Remnants of bone graft particle were observed as hollow pores in decalcified histologic sections (Figure 3).

H/C Block scaffold

Any remnants of H/C block scaffold were not observed and little inflammatory cells were observed. Epithelial apical migration was least. Cementum regeneration were greatest among 3 groups. A little amount of bone was regenerated. (Figure 4) In all groups, Any root resorption or ankyosis were observed. Under limited experimental condition, statistical analysis was not possible.

Histometric analysis

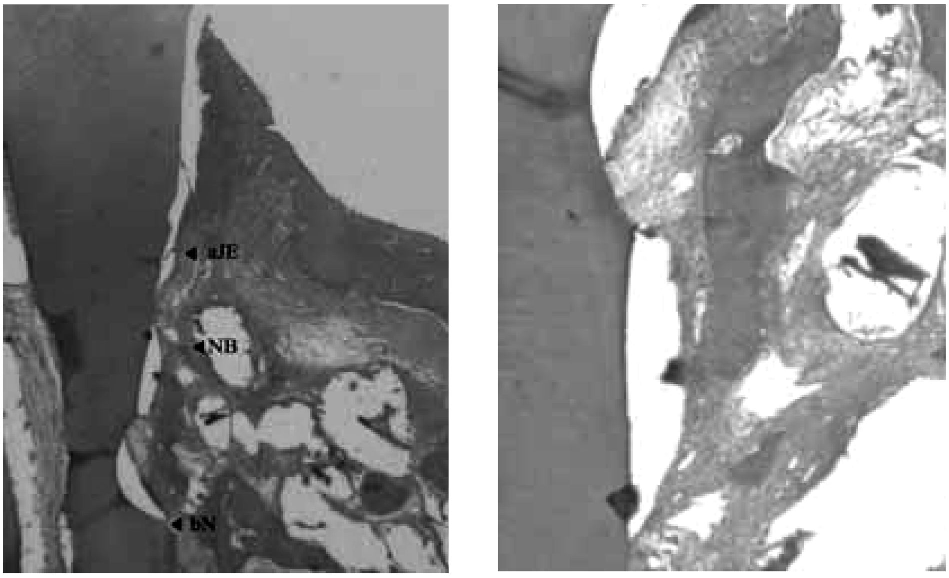
In surgical control group, mean values of regenerated bone, regenerated cementum, connective tissue, and epithelium was 0.33 mm, 0.80 mm, 0.97 mm, and 1.54 mm.

In H/T group, mean values of regenerated bone, regenerated cementum, connective tissue, and epithelium was 0.74 mm, 1.24 mm, 0.60 mm, and 1.22 mm.

In H/C group, mean values of regenerated bone, regenerated cementum, connective tissue, and epithelium was 0.42 mm, 1.33 mm, 0.78 mm, and 1.22 mm. (Table 1)

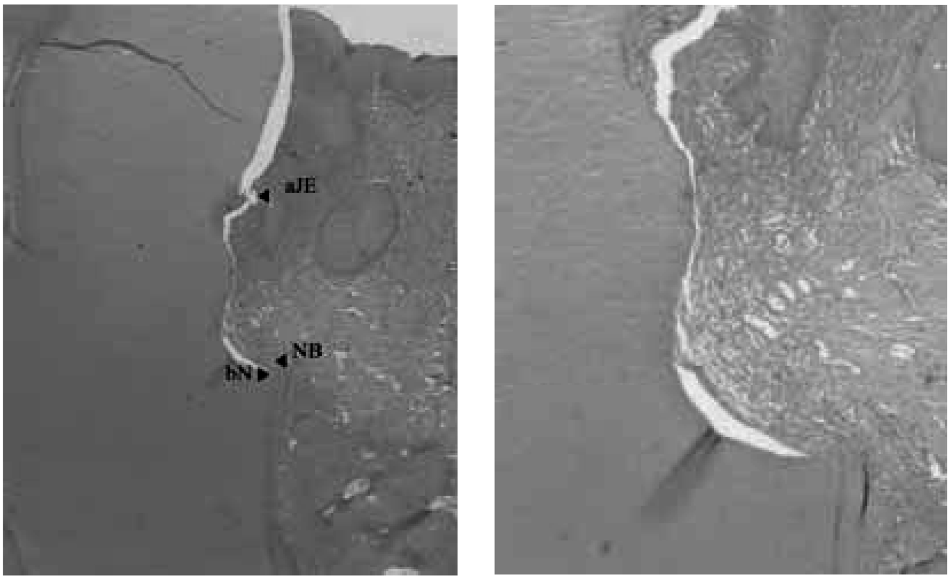
Discussion

The ultimate goal of periodontal therapy is to regenerate the supporting tissue. Various procedures have been developed and applied for regenerative periodontal therapy. It comprises GTR.[13-16] autografts, other bone grafts, and the application of growth factor.[17-19] Regeneration was reported with those tech-



(a) (b)

Figure 3. a: Section of HA/β-TCP particle graft group (× 20), b: HA/β-TCP particle graft (× 40).



(a) (b)

Figure 4. a: HA/Chitosan block scaffold (× 20), b: HA/Chitosan block scaffold (× 40).

Table 1. Comparison between experimental groups (mean ± SE) (N = 3)

	Surgical control	HA/β-TCP particle	HA/Chitosan block
Defect height	3.56 ± 0.50 mm	4.01 ± 0.04 mm	3.54 ± 0.15 mm
Junctional epithelium	1.55 ± 0.12 mm	1.49 ± 0.18 mm	1.22 ± 0.72 mm
Connective tissue	0.97 ± 0.35 mm	0.60 ± 0.19 mm	0.78 ± 0.99 mm
New Bone	0.33 ± 0.34 mm	0.74 ± 0.27 mm	0.42 ± 0.00 mm
New cementum	0.80 ± 0.41 mm	1.24 ± 0.12 mm	1.33 ± 0.53 mm

niques, but perfect regeneration was not achieved yet with these conventional regenerative therapies.

Bone graft for periodontal regeneration has been widely investigated as a graft itself, a space provider and a clot stabilizer for GTR. Autograft has been proposed as a material of choice for regenerative periodontal therapy. However, additional donor site preparation is required for autografts. Limited volume also makes it difficult for autografts to be used for most of the cases. Allografts and alloplasts have been used as alternatives, but it was revealed some of these materials were just biocompatible fillers encapsulated by connective tissue from histologic studies.[20].

Therefore, devising alternative techniques with new materials is required for periodontal regenerative therapy. Proper testing models should also be developed to evaluate the effect a new regenerative material.

One wall infrabony defect was used in this study. The number of bone walls is a critical factor determining treatment outcomes in intrabony periodontal defects. One-, 2-, and 3-wall intrabony periodontal defects were surgically produced at the proximal aspect of mandibular premolars in either right or left jaw quadrants in six beagle dogs.[21] After 8 weeks, Bone and cementum regeneration was positively correlated to the number of bone walls limiting the intrabony periodontal defects. One- wall intrabony defect could be a reproducible model to evaluate candidate technologies for periodontal regeneration.

The animals were sacrificed 8weeks after the experimental surgeries in this study. The healing time is also an important factor for proper study model. Naturally or ligature induced loss of attachment and surgically induced loss of attachment showed no difference in healing. In one-wall defect model, Moon et al. could not discern cementum regeneration until week 6 in a dog intra-bony defect model using light microscopy suggesting that wound maturation must progress over several weeks until cementum formation may be appreciable by light microscopy.[15] Moreover, Choi et al. observed no additional bone and cementum formation between an 8-and 24-week healing interval for sham-surgery controls in a dog intra-bony defect model suggesting longer observation intervals may not be necessary to capture the osteogenic potential and cementogenesis.[19] Therefore, the design of this study seems properly designed based on well documented previous studies.

Naturally derived or synthetic polymers are materials used commonly for bone tissue engineering scaffolds. Polymers can be applied with various types according to specific needs.[6] Collagen, chitosan, and hyaluronic acid are some natural polymers that have been used in bone tissue engineering applications. Chitosan has an excellent potential as a structural base material for a variety of engineered tissue system.[10] Chitosan has been reported to enhance periodontal tissue regeneration.[10-12] In *in vivo* studies, liquid type of chitosan induced bone regeneration in rat calvarial defect models, and 1-wall defect model in dogs. As a bone graft, chitosan was modified with hydroxyapatite and calcium phosphate in order to enhance mechanical properties. Enhanced bone regeneration and cementum regeneration was achieved in dog 1-wall defect model.[12,22,23] As a membrane, Chitosan was fabricated in a membrane form with or without antibiotics. This membrane was tested in rat calvarial model and dog 1-wall defect.[24] Chitosan membrane functions acceptably. However, space maintaining property appears inferior to e-PTFE membrane.

Scaffolds for bone formation may contain inorganic compounds to enhance proliferation. Examples include hydroxyapatite, calcium phosphate cements, metals, and calcium sulfate.[24] Hydroxyapatite is highly biocompatible and serves as the scaffold for the osteogenic precursor cells, promoting their differentiation into osteoblasts.[25] However, there are some disadvantages with these materials. Particulate hydroxyapatite lacks shape and cohesive strength; therefore, it tends to be dislodged and to migrate under externally applied forces during the healing period. For periodontal regeneration, space provision is one of the key factors for successful result. The difference in effectiveness between a block type and a particle type of grafts were evaluated in guided bone regeneration model. However, few studies were done in periodontal defects. A block type of grafts seems to be better in space maintaining than a particle type of grafts in general agreement.

The purpose to fabricate H/C Block scaffold is combination of cementum regeneration of chitosan, high biocompatibility of hydroxyapatite and enhancement of space provision by block type.

The aim of this study was to evaluate the biocompatibility of H/C Block scaffold into 1- wall intrabony defects in the beagle dogs and evaluate effect of block type graft by comparison with particle type graft.

At 8 weeks after implantation of H/C Block scaffold, it did not show any inflammatory infiltrates or any residual remnants of block scaffold. In a histometric analysis, enhancement of cementum regeneration and bone regeneration were observed. From these results, H/C Block scaffold seems to have a good biocompatibility. The time for resorption of material was a concern. Faster resorption of the material would be desirable to avoid the risk of any infection of the residual material during periodontal healing than delayed resorption. For an ideal scaffold for bone regeneration, the time for scaffold's resorption should be similar to that for new bone formation. H/C Block scaffold was resorbed faster than new bone formed in this study. Prolonged space maintaining would be required to enhance new bone formation.

In the aspect of bone regeneration, surgical control group, H/T particle group and H/C block group showed 0.33 ± 0.34 mm, 0.74 ± 0.27 mm, 0.42 ± 0.00 mm. H/T particle group showed the most bone regeneration.

In cementum regeneration, surgical control group, H/T particle group and H/C block group showed 0.80 ± 0.41 mm, 1.24 ± 0.12mm, 1.33 ± 0.53 mm. H/C block group and H/T particle group showed similar result, more than surgical control group.

In apical migration of epithelium, surgical control group, H/T particle group and H/C block group showed 1.55 ± 0.12 mm, 1.49 ± 0.18mm, 1.22 ± 0.72mm. Block type graft showed the least migration. Surgical control and H/T particle group showed similar results.

In connective tissue adhesion, surgical control group, H/T particle group and H/C block group showed 0.97 ± 0.35 mm, 0.60 ± 0.19 mm, 0.78 ± 0.99 mm. The surgical control showed the most value. No root resorption or ankylosis was observed.

In the groups of H/C block scaffold, in a previous study as graft type, bone regeneration was similar to surgical control but cementum regeneration was more than surgical control. Any remnants of H/C block scaffold were not observed at 8 weeks specimens. H/C block scaffold has not functioned as space provider long enough to promote bone regeneration. But chitosan has an effect on enhancing cementum regeneration. More recession was observed than surgical control.[23)]

In H/T particle group, bone regeneration and cementum regeneration were more than surgical control group. Remnants were observed at the specimens. H/T particle remains at 8 weeks. It functions as a space provider long enough to promote bone regeneration. Bone regeneration was more than H/C group with this space maintaining. Recession was similar to surgical control group.

H/C block scaffold functions initially to enhance cementum regeneration due to its chitosan portion but block was absorbed too soon for bone to grow in. Therefore, more recession was followed. At initial healing period, chitosan may enhance cementum regeneration and bone regeneration, but in late healing period the block fails to maintain its space so a little bone regeneration enhancement was observed. Block-type bone substitute is known to maintain space better than particle- type. However, in the present study, H/C block resorbed faster than H/T. The proportion of HA probably affected the resorption rate. The more HA in bone substitute is expected the less resorption during healing. The ratio of HA/chitosan in H/C block was 3 : 7, whereas the ratio of HA/ β-TCP in H/T group was 7 : 3. Therefore, it seems that the resorption of H/C block was faster than H/T particle in the present study. If the proportion of HA in H/C block changed, the result would be different. Comparing two different materials with the same type would be more beneficial to evaluate the difference of effect. Particle-type H/C should be compared with particle-type H/T for more reliable results. In addition, comparing two different materials with the same particle size would be more helpful to evaluate the difference of effect. Therefore, the surface area which affects capability of regeneration can be given equally to each group.

H/T particle maintained its space long enough for bone to grow in. Therefore, bone regeneration in H/T group was more than surgical control and H/C group. However, less cementum regeneration was observed than H/C. Recession was little.

H/T particle had a superior mechanical property for space maintenance than H/C block did. More formation of new bone was observed. The difference in periodontal regenerative effect between block and particle type graft is difficult to draw conclusion in this experimental result.

Conclusion

HA/chitosan block scaffold showed good biocompatibility and cementum regeneration due to effect of chitosan. However, it was less effective in bone regeneration due to rapid resorption. Some modification is needed for scaffold to reduce resorption rate in order to be used as scaffold for periodontal regeneration. For evaluation of difference between block and particle type graft in periodontal regeneration, in this experiment, it was not possible to draw conclusion due to difference in its chemical difference.

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