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Hydrogen-Rich Water for the Treatment of Experimental Peri-Implantitis

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Background: Due to its antioxidant and anti-inflammatory effects, hydrogen-rich water has been used to reduce inflammatory responses in many inflammatory diseases. However, its efficacy in treating peri-implantitis remains unclear. This study aims to investigate the efficacy of hydrogen-rich water in reducing inflammatory responses in experimental peri-implantitis.
Methods: A ligature-induced experimental peri-implantitis model was established using three Beagle dogs (n = 24 implants). The implants were divided into two groups: a hydrogen-rich water-treated group (n = 12 implants), and a non-treated control group (n = 12 implants). The clinical indices, including the gingival index (GI), modified sulcus bleeding index (mSBI), and probing depth (PD), were examined. Hematoxylin and eosin (H&E) staining was used to assess the inflammatory cell infiltrate in the peri-implant tissue. Additionally, three host-derived pro-inflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6), and matrix metalloproteinase-8 (MMP-8) were assessed using enzyme-linked immunosorbent assay (ELISA). Furthermore, microbial community profiles were analyzed using high-throughput sequencing (HTS) technology.
Results: In terms of the GI, the differences between the control and treated groups at 0, 2, and 4 weeks were not statistically significant (all $p > 0.05$). However, the GI value in the treated group at 6 weeks was significantly lower than that in the control group ($p = 0.005$). As for the PD, the differences between the control and treated groups at 0, 2, 4, and 6 weeks were not statistically significant (all $p > 0.05$). Regarding the mSBI, the difference between the control and treated groups at 0 week was not statistically significant (all $p > 0.05$), while the mSBI value in the treated group at 2, 4, and 6 weeks was significantly lower than that in the control group ($p = 0.008$, $p = 0.005$, $p = 0.001$). Compared to the non-treated group, the hydrogen-rich water-treated group showed a significantly lower number of inflammatory cells, lower pro-inflammatory cytokines, IL-1, IL-6, and MMP-8 levels ($p < 0.05$). Additionally, there was a significantly decreased relative abundance of pathogenic bacterial species including *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Fusibacter*, and *Fretibacterium* ($p < 0.05$).
Conclusions: The use of hydrogen-rich water for treating peri-implantitis showed promise and effectiveness within the scope of this study.

Keywords: peri-implantitis; hydrogen-rich water; Beagle dog; high-throughput sequencing; bioinformatics

Introduction

Hydrogen is a nonmetallic, colorless, and odorless diatomic gas, which is the most abundant and lightest element [1]. For a long time, hydrogen was considered a physiologically inert gas due to its weak binding force with haemoglobin compared to that of oxygen, and its inability to be absorbed by the human body in large quantities [1]. In 1975, Dole established squamous cell carcinoma models in mice and placed them in a hydrogen chamber to show improvement in tumor status [2]. By selectively decreasing hydroxyl radical (OH) and peroxynitrite anion, the breathed hydrogen displayed antioxidant and anti-apoptotic characteristics, protecting the brain against ischemia/reperfusion (I/R) injury [3]. Subsequently, the application of hydrogen as an emerging therapeutic method in many diseases, especially due to its anti-apoptosis and anti-oxidation properties has gained significant attention [4]. Hydrogen plays a crucial role in protecting different tissues and organs from antioxidant damage [4]. In periodontal tissues, host cells produce reactive oxygen species (ROS) as part of their defense response [5]. ROS can damage host DNA, proteins, and lipids in normal cells and tissues while targeting invading pathogens [6], leading to local tissue damage [7–

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9]. An imbalance between ROS production and the antioxidant defense system can trigger the oxidative stress response, stimulate osteoclast differentiation, and lead to alveolar bone absorption [10,11]. Therefore, maintaining a balance between ROS and antioxidants is essential for periodontal health [12]. Traditional antioxidant therapies neutralize the harmful and protective effects of ROS simultaneously, while hydrogen, as a weak reducing agent, keeps other beneficial oxidants active by reacting with strong oxidants, thus avoiding this disadvantage [13].

Dental implants are currently widely used to replace missing teeth. Studies have shown a high 10-year survival rate of 89.23%, with a cumulative 16-year survival rate of 82.94% [14], indicating that implant restoration is a relatively predictable procedure. However, peri-implant inflammation is a common biological complication [15]. It can be divided into peri-implant mucositis and peri-implantitis. The clinical symptoms of peri-implant mucositis include redness, swelling, and bleeding on probing [16]. Peri-implantitis is associated with bone resorption, pocket formation, and purulent secretion [17]. The imbalance between osteoblasts and osteoclasts, caused by bacterial products and inflammatory cytokines, is the primary cause of inflammatory bone loss. Various inflammatory signals and cytokines, such as interleukin-6 (IL-6), interleukin-8 (IL-8), prostaglandin E2 (PGE2), and tumor necrosis factor (TNF), can regulate osteoclast activity [18]. Pro-inflammatory cytokines IL-6, IL-8, and TNF in peri-implantitis-affected tissues are reportedly higher than those in periodontitis [19]. Overall, peri-implantitis is a heterogeneous mixed infection with a distinct microbial composition [20]. Biopsies of human peri-implant lesions show twice as large inflammatory infiltrates as compared to periodontitis [21]. In peri-implantitis, a higher number of plasma cells, macrophages, and neutrophils, and greater density of the vascular are evident [21]. Levels of matrix metalloproteinases (MMPs) are higher in peri-implantitis as compared to chronic periodontitis and healthy gingival tissue. Peri-implantitis can also progress faster, leading to more severe bone loss [22].

Peri-implant disease and periodontal disease have similar clinical characteristics, etiology, and treatment. For peri-implant mucositis, non-surgical treatments seem to be enough, mainly comprising the removal of biofilm and calculus from the implant surface through mechanical debridement, as well as chemical plaque control [16]. However, peri-implantitis requires surgical treatment and debridement of the implant surface has been attempted by chemical agents, air abrasion, or ultrasound [23]. Meta-analysis of adjunctive measures to non-surgical and surgical therapy for peri-implant diseases including glycine powder, air polishing, photodynamic and laser therapy, local and systemic antibiotics, probiotics, ozone therapy, and implantoplasty, have shown heterogeneous clinical outcomes [23]. However, the prognosis of severe peri-implantitis remains unpredictable. Here, we explored the use of hydrogen-rich water to treat peri-implantitis, determine inflammatory mediator levels in peri-implant tissue, and measure the proportion of pathogenic bacterial genera, to provide the experimental basis for a novel treatment modality for peri-implantitis.

Materials and Methods

Experimental Animals

The animal experiment protocols received approval from the Nanfang Hospital Animal Ethics Committee (NFYY-2020-0351). Measures were taken to minimize the number of experimental animals and their suffering. Three healthy adult male Beagles, aged between 12 and 14 months, weighing between 11 and 13 kg, were chosen for the study. They were in good health, with all biochemical measures falling within standard ranges, indicating no signs of infectious or acute systemic diseases. A standard oral examination revealed completely erupted permanent teeth. All experimental procedures adhered to ethical principles and were conducted at the Nanfang Hospital's animal experiment center.

Construction of the Peri-Implantitis Model

The Beagle dogs were fed standard soft food once a day for one week at room temperature of 18 °C. Food intake ceased 24 h before surgery, while water intake ceased 4 h before surgery. The dogs were anesthetized intravenously with 3% pentobarbital sodium at a dose of 30 mg/kg. Anaesthesia was administered via the anterior tibial vein of the hind limb, and sufficient depth of anaesthesia was verified by no response to toe pinching, no response to corneal reflexes, and increased breathing depth and respiration rate. The surgical area was disinfected with 0.5% povidone-iodine and 75% ethanol. Eight teeth were minimally invasively extracted after local infiltration anaesthesia (n = 8 teeth per dog). For implant placement, the pilot drill was used to form 8.5 mm depth osteotomies, and 4.0 mm by 8.0 mm Dentium implants (Dentium Co., Ltd. Company; Gangnam-gu Seoul, Seoul, Republic of Korea) were slowly screwed in and placed 0.5 mm subcrestally. During the surgery, a large amount of cold normal saline was used to rinse the bone. Then, 800,000 U of penicillin was injected before and after the operation. Once the implants were successfully integrated, each implant was wrapped three times with 4-0 silk threads, which were inserted into the depth of the peri-implant sulcus using a probe. The thread was removed when the alveolar bone had absorbed 40% of the implant length.

Different parameters including gingival index (GI), probing depth (PD), and modified sulcus bleeding index (mSBI), were assessed at the baseline (0 w), 2 weeks after the first administration (2 w), 4 weeks after the second administration (4 w), and 6 weeks after the third administration (6 w) by a single examiner at the Animal Cen-

ter of Nanfang Hospital, affiliated with Southern Medical University. This evaluation aimed to measure the interval changes and severity of the inflammation. The GI, which measures gingival inflammation around an implant, was assessed in four gingival units (mesial and distal papillary units and labial and lingual marginal units) using a periodontal probe with a 0.5 mm diameter tip and a force of 25 N. The GI assesses the presence or absence of bleeding on gentle probing, redness, edema, and ulceration of the gingival tissue around an implant, utilizing the following scoring system: 0 = normal gingiva; 1 = mild inflammation (slight change in color, slight edema, no bleeding on probing); 2 = moderate inflammation (redness, edema, and glazing, or bleeding on probing); 3 = severe inflammation (marked redness and edema, tendency toward spontaneous bleeding, ulceration) [24]. PD is defined as the distance between the bottom of the pocket and the peri-implant mucosal margin [25]. In general, a PD associated with peri-implant health should be $\leq$5.0 mm, while a PD $\geq$6 mm is indicative of peri-implantitis [26]. The mSBI is a simple method that records the presence of bleeding in a dichotomous manner [27]. The criteria for scoring the mSBI are as follows: 0 = healthy-looking papillary and marginal gingiva no bleeding on probing; 1 = healthy-looking gingiva, bleeding on probing; 2 = bleeding on probing, change in color, no edema; 3 = bleeding on probing, change in color, slight edema; 4 = bleeding on probing, color change, obvious edema; 5 = spontaneous bleeding, change in color, marked edema [28].

Determination of Hydrogen-Rich Water Concentration

Saturated hydrogen-rich water was prepared by a hydrogen generator and filled into a 6 mL measuring cup. The hydrogen-rich water was incubated at room temperature for 1 min, 10 min, 30 min, 60 min, and 90 min, respectively. The hydrogen content was determined by the methylene blue reductant-oxidant (REDOX) reaction with hydrogen. The titration liquid was added to a cup, shaken evenly, and poured until the hydrogen-rich water turned into a stable positive blue color. The number of titration liquid drops added was counted to determine the concentration of dissolved hydrogen. One drop of titration liquid indicated 100 parts per billion (PPB) of dissolved hydrogen in water.

Clinical Indices Measurements

The left mandible area was set as the treated group, receiving hydrogen-rich water (n = 4 implants per dog $\times$ 3 dogs = 12 implants), while the right mandible area was designated as the control group and rinsed with drinking water (n = 4 implants per dog $\times$ 3 dogs = 12 implants). Hydrogen-rich water from a 50 mL syringe was used for rinsing twice a day for 5 min each time. GI, mSBI, and PD were measured at weeks 0, 2, 4, and 6.

Changes in Inflammatory Factors in Peri-Implant Tissues

Quantitative Analysis of Inflammatory Factors and MMP-8

A Whatman#3 filter paper was inserted into the mesial, distal, buccal, and lingual aspects of the implant for 30 s each to collect the peri-implant crevicular fluid. Subsequently, the filter paper was stored in an Eppendorf tube and sealed in a –80 °C refrigerator. Any papers contaminated with blood or saliva were discarded. This procedure was repeated at weeks 0, 2, 4, and 6 after starting hydrogen-rich water treatment. The expression levels of IL-1, IL-6, and matrix metalloproteinase-8 (MMP-8) were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Bang Yi Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The 96-well ELISA plates were incubated with specific antibodies (1/4000 in 1% BSA/PBS) at 37 °C for 1 h. Then, the plate was treated with ABTS peroxidase. After reading with the Labsystems Multiskan MS 352 Microplate Reader (catalogue No.: MS 352; Brand: Thermo Fisher Scientific; Manufacturer: Labsystems, San Marcos, CA, USA) at a wavelength of 450 nm, the concentrations of IL-1, IL-6, and MMP-8 were calculated using a standard curve established.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) consists of three main steps: total RNA extraction via reverse transcription, cDNA extraction via reverse transcription, and qPCR amplification of the target sequence. Trizol solution was used to extract total RNA from approximately 2 mm bone tissue around the implant. The reverse transcription and cDNA synthesis were conducted using EasyScript First-Strand cDNA Synthesis SuperMix (Catalog number: AE301-02; Transgenbiotech, Beijing, China). Subsequently, quantitative PCR by qPCR amplification was carried out using SYBR Green PCR Master Mix (Catalog number: 4927004; Thermo Fisher Scientific, Waltham, MA, USA). The cycling conditions involved 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. Upon completion of the reaction, the fluorescence signal was automatically analyzed by the Applied Biosystems PRISM® 7500 FAST Sequence Detection System (SDS) software (version 1.5.1, Applied Biosystems, Foster City, CA, USA) and converted into a Ct value. To determine the relative expression of the target gene, the following formula was employed: Target Gene Relative Expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct represents the difference in ΔCt values between the treated group and control group. ΔCt in any group is calculated by subtracting the Ct value of the reference gene from the Ct value of the target gene. The sequences of the forward and reverse primers of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and three examined genes (*MMP-8*, *IL-1*, and *IL-6*) are provided in Table 1.

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Table 1. Forward and reverse primer sequences of the housekeeping gene (*GAPDH*) and three examined genes (i.e., *MMP-8*, *IL-1*, and *IL-6*) for performing RT-qPCR assay (n = 9).

Gene symbol	Primer	Primer Sequence (5′-3′)
GAPDH	Primer-F	CAAGGCTGTGGGCAAGGTCATC
	Primer-R	TTCTCCAGGCGGCAGGTCAG
MMP-8	Primer-F	CTGCTGCTGCTGCTGCTCTG
	Primer-R	GACCATCTCCACATCTTGCTCCTG
IL-1	Primer-F	TGTGAAGTGCTGCTGCCAAGAC
	Primer-R	TACAGAGCTGGTGGGAGACTTGC
IL-6	Primer-F	TGACCACTCCTGACCCAACCAC
	Primer-R	GACTCCGCAGGATGAGGTGAATTG

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *MMP-8*, matrix metalloproteinase-8; *IL-1*, interleukin-1; *IL-6*, interleukin-6; RT-qPCR, Real-Time Quantitative Polymerase Chain Reaction.

Observation of the Inflammatory Cells in Gingival Tissue by Hematoxylin and Eosin (H&E) Staining

After anesthesia, a tissue block measuring 1.0 cm by 1.5 cm by 0.2 cm was resected, containing the implant, and fixed with 4% paraformaldehyde. The tissue block underwent graded ethanol dehydration (70% to 100%) and was then treated with xylene for 30 min until it became transparent. Subsequently, it was permeabilized by soaking in paraffin for 3 h. A wax solution was injected into an abrasive tool, and the tissue block was quickly placed horizontally into the wax solution and then moved to a cooling table for solidification. Next, tissue slices were placed flat on glass slides, and a 30% alcohol aqueous solution was added until the tissues were completely unfolded. They were then transferred to a thermostatic water heater at 56–60 °C for 30–60 min. Finally, the wax slices were placed in a drying oven for 1 h, dewaxed with xylene, and washed successively with 100%, 95%, 90%, and 80% ethanol for 3 min each to remove the xylene. After soaking in distilled water for 5 min, the slices were dried by shaking and then dyed with hematoxylin for 15 min, followed by washing in distilled water for 5 min. The slices were treated with hydrochloric acid alcohol for 2 s, washed with water, dyed with eosin for 15 min, and washed with distilled water for 5 min. Finally, the slices were dehydrated with an ethanol gradient (70%, 80%, 90%, 95%) for 3 min at each concentration, then sealed with neutral gum for observation under a microscope.

Bacterial Profiling of the Peri-Implant Crevicular Fluid by High-Throughput Sequencing

In the 6th week after initiating hydrogen-rich water treatment, peri-implant crevicular fluid was collected as previously described. DNA extracted from the samples was analyzed using agarose gel electrophoresis and quantified with an ultraviolet (UV) spectrophotometer. Then, 16s rRNA primers were designed, and a sample-specific barcode sequence was added for PCR amplification of the ribosomal ribonucleic acid (rRNA) gene. The amplified product was separated by 2% agarose gel electrophoresis, and the target fragments were excised and purified. The purified products were quantified by fluorescence and mixed proportionally according to the sequencing requirements of each sample. Subsequently, sequencing libraries were prepared, and the fragments were selected and purified. Qualified libraries were then diluted and mixed according to the specified requirements. High-throughput sequencing was performed using Illumina MiSeq, and bioinformatic analysis was conducted using the 16S RNA Amplicon Data Analysis (DADA2) package (version 3.16, https://www.bioconductor.org/packages/release/bioc/html/dada2.html).

Statistical Analysis

IBM SPSS Statistics 22.0 (Version 22.0, IBM Corp., Armonk, NY, USA) was used for statistical analysis. The measurement data are presented as mean ± standard deviation ($\bar{x} \pm s$). An independent samples *t*-test was used to compare two groups, repeated analysis of variance was employed to assess changes in each index, and a post hoc Least-Significant Difference (LSD) test was conducted for comparisons between two-time points. Statistical significance was set at a *p*-value below 0.05.

Results

Construction of Peri-Implantitis Model

The PD index was monitored using a periodontal probe every two weeks. If PD exceeded 5 mm with concomitant bleeding, X-rays were obtained to analyze marginal bone resorption. The average probing depth was about 6.5 mm (Fig. 1a), meeting the clinical diagnostic standard for peri-implantitis. There was evident redness of the soft tissue and visible plaque at the exposed implant (Fig. 1b). The peri-implant tissue appeared red and swollen (Fig. 1c). Purulent discharge from the peri-implant sulcus was observed (Fig. 1d).

Determination of Hydrogen-Rich Water Concentration

The titration experiment revealed that the concentration of hydrogen-rich water was the highest within 1 min (instant preparation), with concentrations reaching 1.67 mg/L. The requirements of saturated hydrogen-rich water include a hydrogen content greater than 1.6 mg/L. The concentration reached 1.6 mg/L at a time point close to 5 min, leading to the conclusion that hydrogen-rich water should be used immediately within 5 min after preparation (Fig. 2). The results of repeated measure analysis of variance indicated a statistically significant difference in the time main effect ($F = 528.842$; $p < 0.001$). Furthermore, the results

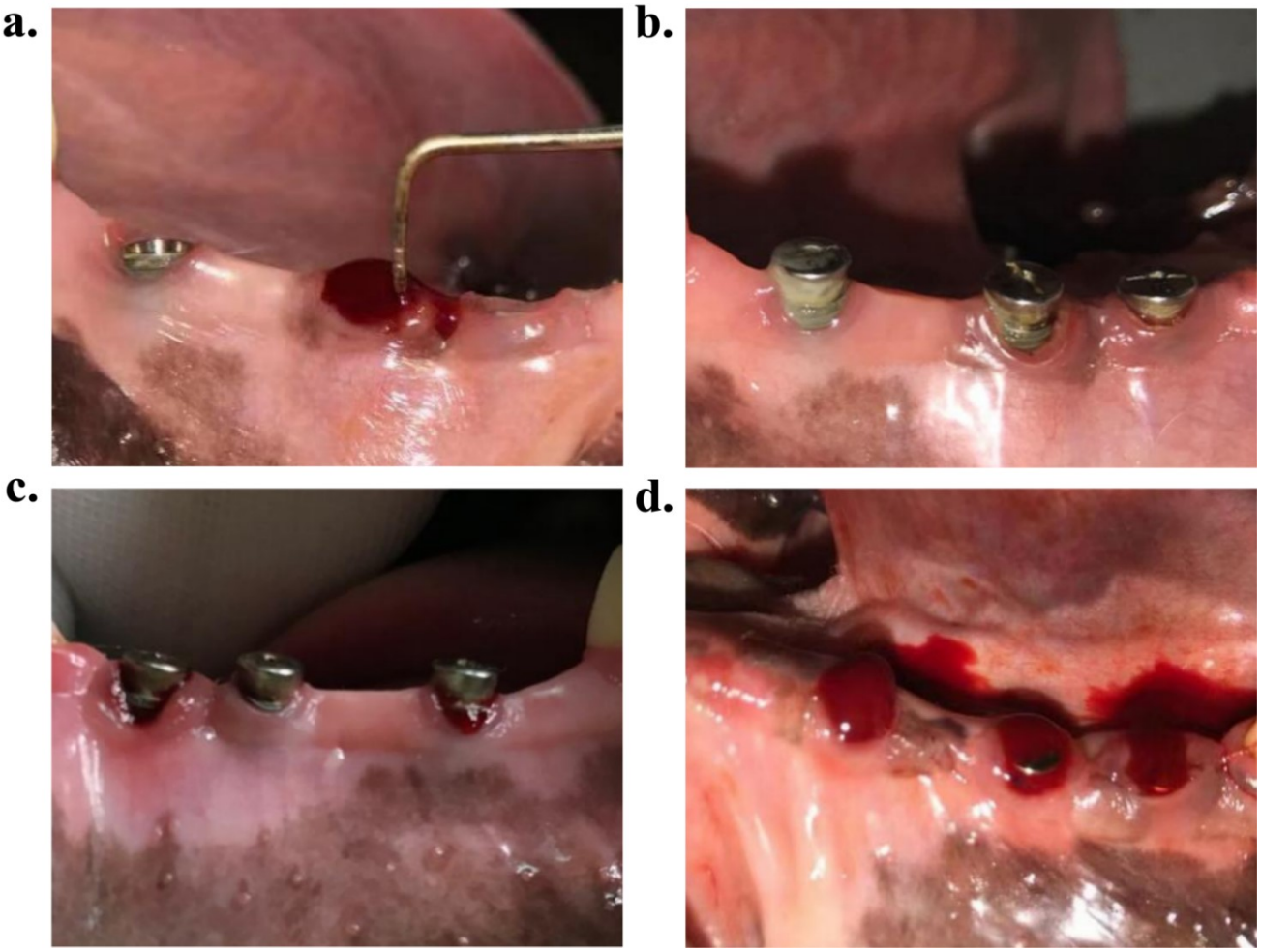

Fig. 1. Clinical images of peri-implantitis lesions established in Beagle dogs. (a) Bleeding on probing. (b) Recession and implant exposure. (c) The peri-implant tissue appeared red and swollen. (d) Purulent discharge from the peri-implant sulcus.

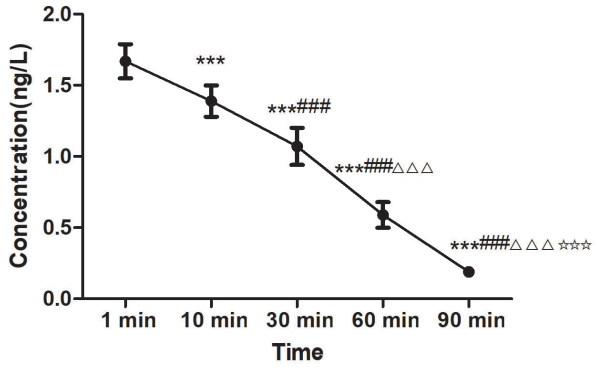

Fig. 2. Change in hydrogen-rich water concentration over time, recorded over a 90 min period. The error bars represent the standard deviation of measurements for titration concentration in three separate sample runs (n = 12). ***$p < 0.001$ *vs* 1 min; $^{###}p < 0.001$ *vs* 10 min; $^{△△△}p < 0.001$ *vs* 30 min; ☆☆☆$p < 0.001$ *vs* 60 min.

of the time point comparisons revealed that the differences between 10, 30, 60, and 90 min compared to 1 min were all statistically significant (p = 0.000, 0.000, 0.000, 0.000). Likewise, the differences between 30, 60, and 90 min compared to 10 min were all statistically significant (p = 0.000, 0.000, 0.000). Similarly, the differences between 60 min and 90 min compared to 30 min were all statistically significant (p = 0.000, 0.000). Additionally, the difference between 90 min and 60 min was also statistically significant (p = 0.000).

Clinical Indices

Fig. 3 shows the mean score ± standard deviation (SD) of GI, PD, and mSBI at baseline (0 w), 2 weeks (2 w), 4 weeks (4 w), and 6 weeks (6 w) in two different groups (treated group and control group) (n = 12 implants per group). As shown in Fig. 3, with the progression of hydrogen-rich water treatment, GI and mSBI gradually decreased in the treated group.

The analysis of measurement variance for the GI index is presented in Fig. 3a. The main effect difference of

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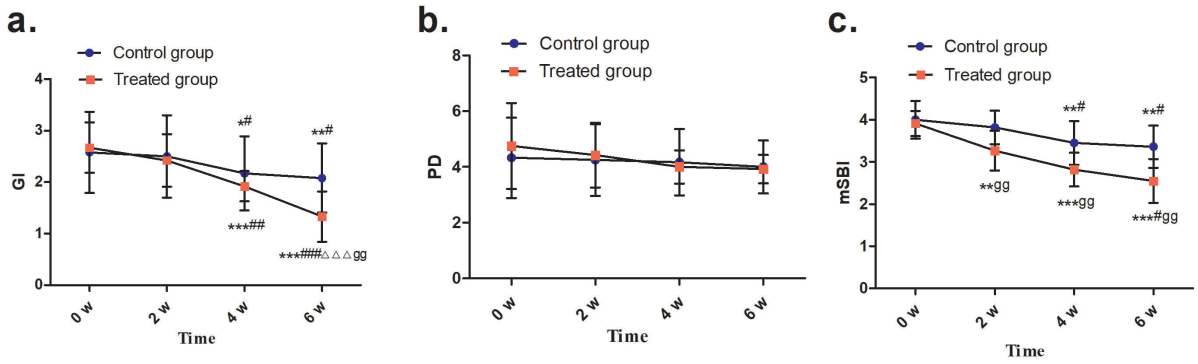

Fig. 3. Change in clinical indices over time, recorded over 6 weeks. (a) GI. (b) PD. (c) mSBI. The error bars represent the standard deviation of measurements for GI, PD, and mSBI (n = 12). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* 0 week; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* 2 weeks; $^{\triangle\triangle\triangle}p < 0.01$ *vs* 4 weeks; $^{gg}p < 0.01$ *vs* control group. GI, gingival index; PD, probing depth; mSBI, modified sulcus bleeding index.

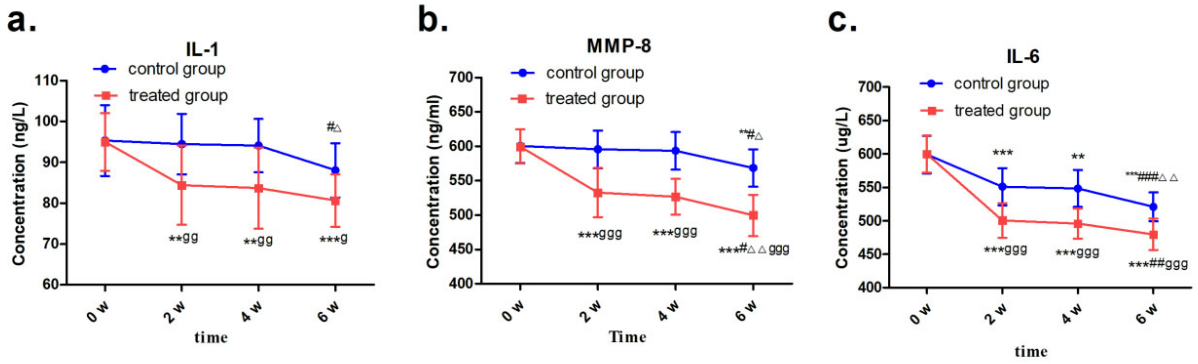

Fig. 4. Changes of immuno-inflammatory mediators in peri-implant crevicular fluid over time, recorded over 6 weeks. (a) IL-1. (b) IL-6. (c) MMP-8. The error bars represent the standard deviation of measurements for IL-1, IL-6, and MMP-8 (n = 12). $^{**}p < 0.01$, $^{***}p < 0.001$ vs 0 week; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* 2 weeks; $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ *vs* 4 weeks; $^{g}p < 0.05$, $^{gg}p < 0.01$, $^{ggg}p < 0.001$ *vs* control group.

the GI grouping was not statistically significant ($F = 1.303$; $p = 0.266$). However, the main effect difference of time was statistically significant ($F = 32.560$; $p < 0.001$). Additionally, there was a significant interaction effect between time and grouping ($F = 6.160$; $p = 0.001$). Consequently, further simple effect analysis was conducted, controlling for the grouping factor to compare the time points. The results revealed statistically significant differences for the control group at 4 and 6 weeks compared to the control group at 0 week ($p = 0.017$, 0.007), and statistically significant differences for the control group at 4 and 6 weeks compared to the control group at 2 weeks ($p = 0.039$, 0.017). Furthermore, the treated group exhibited statistically significant differences at 4 and 6 weeks compared to the 0 week ($p = 0.000$, 0.000), and at 4 and 6 weeks compared to the 2 weeks ($p = 0.007$, 0.000). Additionally, the treated group showed statistically significant differences at 6 weeks compared to the 4 weeks ($p = 0.000$).

Regarding the repeated analysis of measurement variance for the PD index, the results in Fig. 3b show that the main effect of group difference is not statistically significant ($F = 0.038$; $p = 0.847$), the main effect of time difference is statistically significant ($F = 5.837$; $p = 0.012$), but there is no interaction effect between time and group ($F = 1.497$; $p = 0.238$).

The repeated analysis of measurement variance for the mSBI index, revealed statistically significant main effect differences for both the main effect ($F = 16.429$; $p = 0.000$) and time ($F = 29.020$; $p < 0.001$) as shown in Fig. 3c. Similarly, an interaction effect between time and grouping was observed ($F = 3.492$; $p = 0.021$). Subsequently, further simple effect analysis was conducted, controlling for the time factor to compare between the groups. The results indicated no statistically significant difference between the control and treated groups at 0 week ($p > 0.05$). However, statistically significant differences were observed be-

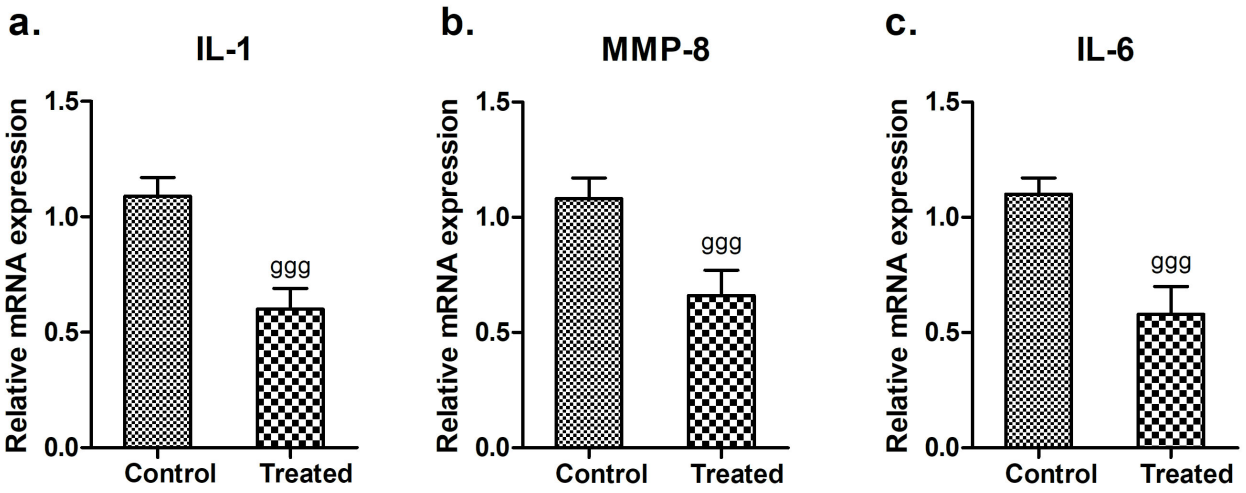

Fig. 5. The relative mRNA expression of the immuno-inflammatory mediators in peri-implant surrounding bone tissue, recorded at the 6 weeks time point. (a–c) The error bars represent the standard deviation of measurements for IL-1, IL-6, and MMP-8 (n = 12). $^{ggg}p < 0.001$ *vs* control group.

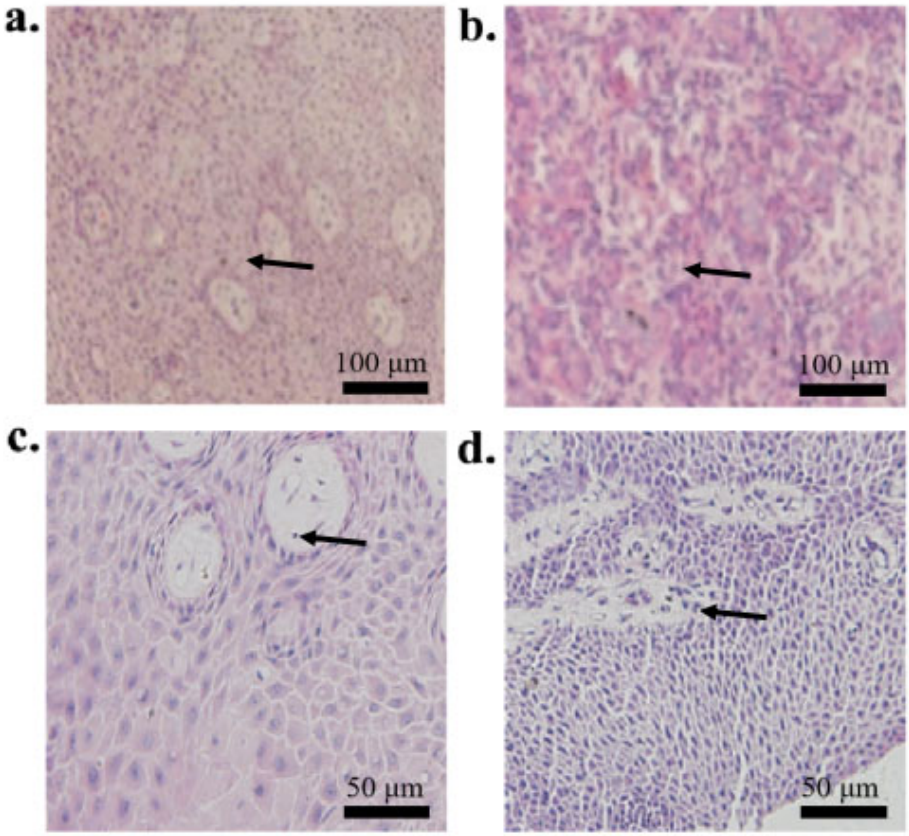

Fig. 6. Hematoxylin and eosin (H&E) staining of peri-implantitis soft tissue. (a) The control group displays stratified squamous epithelial tissue, showing scattered inflammatory cell infiltration, indicated with black arrows (Total magnification: 10×) (Scale bar = 100 µm). (b) The treated group exhibits the same stratified squamous epithelium, with less inflammatory cell infiltration, predominantly lymphocytic, as indicated with black arrows (Total magnification: 10×) (Scale bar = 100 µm). (c) The control group exhibits infiltration, with a predominance of lymphocytes, as indicated with black arrows (Total magnification: 20×) (Scale bar = 50 µm). (d) The treated group presents stratified squamous epithelium, with less inflammatory cell infiltration, predominantly lymphocytic, as indicated with black arrows (Total magnification: 20×) (Scale bar = 50 µm) (n = 12).

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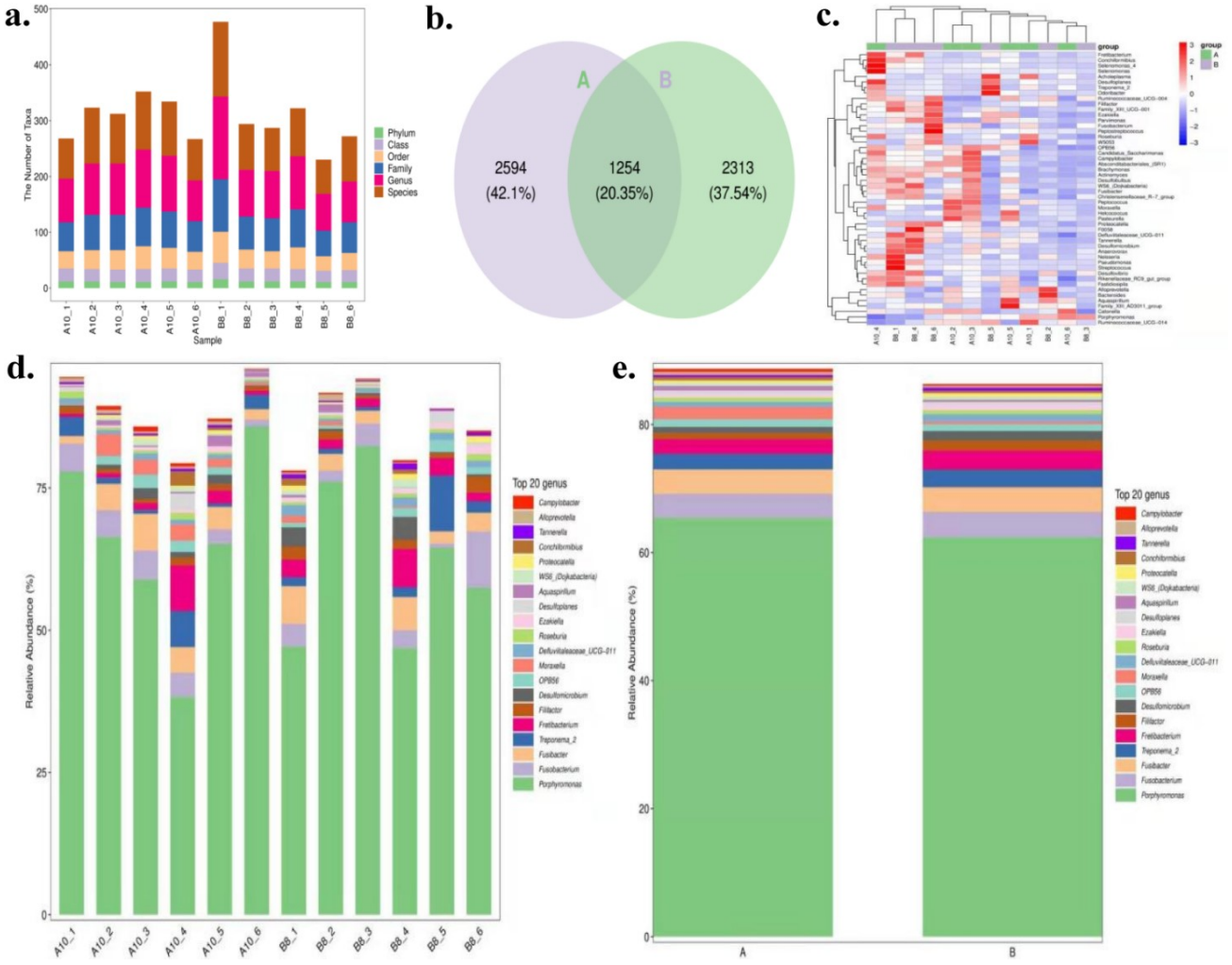

Fig. 7. The results of high throughput sequencing. (a) The number of microbial taxa at each level. (b) Venn diagram of ASV/OTU of the groups (A: control group; B: treated group). (c) Heat map of species composition at the genus level of biclustering. (d) Histogram of species composition at the genus level (comparison among samples). (e) Histogram of species composition at the genus level (comparison between groups) (A: control group; B: treated group) (n = 6).

tween the control and treated groups at each other time point (i.e., 2 weeks, 4 weeks, and 6 weeks) (p = 0.008, 0.005, 0.001). When controlling for the grouping factor, the comparison of the control group at 4 weeks and 6 weeks with 0 week showed statistically significant differences (p = 0.006, 0.002); and the comparison of the control group at 4 weeks and 6 weeks with 2 weeks showed statistically significant differences (p = 0.038, 0.016). Moreover, the treated group exhibited statistically significant differences at 2 weeks, 4 weeks, and 6 weeks when compared with 0 week (p = 0.002, 0.000, 0.000), and a significant difference was observed for the treated group between 6 weeks and 2 weeks (p = 0.012).

Changes in Inflammatory Factors in Peri-Implant Tissues

Quantitative Analysis of Immuno-Inflammatory Mediators by ELISA

Regarding IL-1, the main effect difference for the group was statistically significance (F = 9.144; p = 0.006), as was the main effect difference for time (F = 11.807; p < 0.001). Additionally, there was an interaction effect between time and group (F = 3.273; p = 0.026) (Fig. 4a). Consequently, further analysis of simple effects was conducted, controlling for the factor of time and comparing the results between groups. It was found that there was no statistically significant difference between the control group and the treated group at 0 week (p > 0.05). However, there were statistically significant differences between these two groups at 2 weeks, 4 weeks, and 6 weeks (p = 0.009, 0.006, 0.011, respectively). Controlling for the factor of group and comparing different time points, significant differences

were observed for the control group at 6 weeks when compared to the control group at 2 weeks and 4 weeks (p = 0.041, 0.014, respectively). Significant differences were also observed for the treated group at 2 weeks, 4 weeks, and 6 weeks when compared with the treated group at 0 week (p = 0.003, 0.004, 0.000, respectively).

Regarding MMP-8, the main effect difference for the group was statistically significant (F = 44.939; $p < 0.001$), as was the main effect difference for time (F = 28.833; $p < 0.001$). Additionally, there was an interaction effect between time and group (F = 10.603; $p < 0.001$) (Fig. 4b). Consequently, further analysis of simple effects was conducted, controlling for the factor of time and comparing the results between groups. It was found that there was no statistically significant difference between the control and treated groups at 0 week ($p > 0.05$). However, statistically significant differences were observed between these two groups at 2 weeks, 4 weeks, and 6 weeks (p = 0.000). When controlling for the factor of group and comparing different time points, significant differences were observed for the control group at 6 weeks when compared with the control group at 0 week, 2 weeks, and 4 weeks (p = 0.008, 0.042, 0.032, respectively). Similarly, significant differences were observed for the treated group at 2 weeks, 4 weeks, and 6 weeks compared to the treated group at 0 week (p = 0.000). Furthermore, significant differences were observed for the treated group at 6 weeks when compared with the treated group at 2 weeks and 4 weeks (p = 0.018, 0.003, respectively).

Regarding IL-6, the statistical analysis revealed significant main effects for both group (F = 26.795; $p < 0.001$) and time (F = 89.608; $p < 0.001$), as well as an interaction effect between time and group (F = 7.465; p = 0.001) (Fig. 4c). Subsequent simple effects analysis, controlling for the factor of time, showed no significant difference between the control and treated groups at 0 week ($p > 0.05$). However, significant differences were observed at 2 weeks, 4 weeks, and 6 weeks (p = 0.000). Controlling for the factor of group, significant differences were observed for the control group at each time point (2 weeks, 4 weeks, and 6 weeks) compared to 0 week (p = 0.000, 0.003, 0.000, respectively). Additionally, significant differences were observed for the control group at 6 weeks compared with the control group at 2 weeks and 4 weeks (p = 0.000, 0.005, respectively). Significant differences were also observed for the treated group at 2 weeks, 4 weeks, and 6 weeks when compared with the treated group at 0 week (p = 0.000), as well for the treated group at 6 weeks compared to the treated group at 2 weeks (p = 0.007).

The mRNA Expression of Immuno-Inflammatory Mediators by qRT-PCR

Fig. 5 shows the relative mRNA expression levels of three immuno-inflammatory mediators (IL-1, IL-6, and MMP-8) in the surrounding tissues of the treated and control groups. The mRNA expression of these three mediators in the treated group was significantly lower compared with that in the control group ($p < 0.001$).

Gingival Tissue Staining Section

The histological examination of Beagle dog gingival epithelial tissues was conducted using hematoxylin and eosin (H&E) staining to compare control and treated groups. Fig. 6a,c represent the control group, showcasing stratified squamous epithelial tissues. Fig. 6a displays scattered inflammatory cell infiltration, highlighted by black arrows. Fig. 6c also exhibits similar infiltration, with a predominance of lymphocytes. Conversely, Fig. 6b,d illustrate the drug treatment group, showing the same stratified squamous epithelium, but with noticeably less inflammatory cell infiltration, predominantly lymphocytic, as indicated by black arrows. This comparison between control and treated tissues provided insights into the impact of Hydrogen-rich water on gingival inflammatory responses.

Bacterial Abundance in the Peri-Implant Crevicular Fluid

Fig. 7a shows the taxa abundance (i.e., phylum, class, order, family, genus, and species) in different samples of treated and control groups. The DADA2 pipeline was used to identify differences between the groups. The overlapping area of the ellipse (group) illustrates shared bacteria between treated and control groups (Fig. 7b). Fig. 7c shows samples with UPGMA clustering based on Euclidean distances using the species composition data. A red block indicates that the bacterial genus was more abundant in this sample than in others, while the blue block indicates lower abundance. The bacterial abundance in the control group was higher than that in the treated group. The relative abundance of each bacterial genus was determined, and the top 20 were depicted (Fig. 7d). In comparison with the control group, the relative abundance of several pathogenic genera (e.g., Porphyromonas, Fusobacterium, and Fusibacter) was significantly lower in the treated group, with statistical significance ($p < 0.05$) (Fig. 7e).

As shown in Table 2, the results of the independent samples t-test indicated that the relative abundance of two bacterial genera *Porphyromonas* (p = 0.010) and *Fusobacterium* (p = 0.002), was significantly reduced in the treated group compared with the control group. Additionally, *Fusibacter* (p = 0.012) and *Fretibacterium* (p = 0.022) also showed significant reductions in the treated group. On the other hand, there was no significant difference in the relative abundance of the other two bacterial genera, *Treponema-2* (p = 0.172) and *Filifactor* (p = 0.299), between the control group and the treated group.

After hydrogen-rich water treatment for peri-implantitis, *Porphyromonas*, *Fusobacterium*, and *Fusibacter*, which are major pathogenic bacterial genera, significantly decreased in the peri-implant crevicular fluid. A

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Table 2. Relative abundance of selected bacterial genera ($\bar{x} \pm s$, n = 6).

	Control group (n = 6)	Treated group (n = 6)	*t*	*p*
Porphyromonas	0.671 ± 0.068	0.540 ± 0.074	3.193	0.010
Fusobacterium	0.047 ± 0.003	0.032 ± 0.008	4.300	0.002
Fusibacter	0.037 ± 0.011	0.022 ± 0.005	3.041	0.012
Treponema-2	0.019 ± 0.003	0.016 ± 0.004	1.470	0.172
Fretibacterium	0.024 ± 0.006	0.016 ± 0.004	2.717	0.022
Filifactor	0.006 ± 0.002	0.004 ± 0.004	1.095	0.299

previous report demonstrated that hydrogen-rich water caused a significant decrease in *in vitro* streptococcal biofilm formation and the mRNA expression of glucosyltransferases and glucan-binding proteins [29]. The use of hydrogen-rich water treatment is speculated to have an antibacterial effect, which may promote the resolution of peri-implantitis.

Discussion

The present study investigated the anti-inflammatory and anti-microbial effects of hydrogen-rich water on peri-implantitis in a dog model. The study showed significant treatment effects, including histological, microbiological, and immunological. Dogs were chosen due to their large size, strong surgical operability, docile personality, and easy control, making them a suitable choice for experimental animals in constructing peri-implantitis models [30]. Previous research has shown that the shape and size of bone defects resulting from the silk ligation technique around the implant in dogs are comparable to lesions that develop naturally in humans [31]. In addition, the distribution of supragingival and subgingival plaque bacteria associated with periodontitis in Beagles was observed to be similar to that in human periodontitis [32]. Additionally, peri-implantitis has been identified as a condition caused by microorganisms in chronic periodontitis [31]. Therefore, a dog model was applied in the present study.

Here, we performed immediate implant placement after tooth extraction, which reduced the experimental duration and placed the implants in the distal premolar socket. The silk ligation method was used to induce peri-implantitis, a widely applied and predictable technique. To shorten the modeling time, high-sugar soft food feeding was also implemented. Within the initial 4 weeks, mechanical stimulation around the implant significantly promoted soft tissue swelling and gingival bleeding. After the fifth week, the gingival bleeding index significantly increased. Clinically, plaque accumulation and pronounced oedema were observed. Moreover, there was a significant increase in marginal bone resorption, and implant thread exposure was evident upon probing, indicating characteristic peri-implantitis [33]. Peri-implantitis is clinically defined by the presence of redness, mucosal swelling, bleeding and/or suppuration, deepening of the periodontal pocket near the implant, and loss of supporting bone [21].

The hydrogen molecule is very small and rapidly dissipates. Consequently, the concentration of hydrogen-rich water stored in a container can quickly decrease to a non-saturated state, making it preferable to use immediately after preparation. Hydrogen-rich water is extremely unstable, necessitating immediate preparation before application to maintain its saturation. It should be handled as gently as possible without shaking, and our findings indicate that it should be used within 5 min after preparation to maintain the optimum concentration.

In addition to the peri-implant examination, radiographic imaging assessment can aid in the diagnosis of peri-implantitis [13]. A study showed that the clinical manifestations of the control group were not significantly improved, the soft tissues were still soft and red, and bleeding was more during probing. Although the treated group was not completely healed, significant improvement was notable. The clinical indications across the groups did not differ significantly, according to statistical analysis. Insufficient sample size and short treatment time for peri-implantitis, and most importantly, the lack of mechanical treatment, which is the mainstay of peri-implant disease management, could account for these results. Mechanical debridement was not applied, as the goal was to isolate the effects of hydrogen-rich water treatment and avoid confounding by other treatment effects.

The formation of bacterial biofilms is considered a crucial initial stage in the development of peri-implant illness. However, the tissue damage caused by peri-implantitis is the result of an immune inflammatory response brought by bacterial stimulation [34]. Cytokines are widely studied biomarkers in peri-implant diseases due to their significance in cellular and vascular inflammatory responses [35]. The production of interleukins (ILs), a group of proinflammatory cytokines produced by polymorphonuclear leukocytes, osteoclasts, and fibroblasts, is essential for the remodeling and turnover of bone [36]. These markers include IL-1β, IL-2, IL-6, and IL-8, which can be detected in gingival crevicular fluid [37]. IL-1 is a stimulatory factor derived from connective tissue catabolism. It can activate fibroblasts and immunize nucleated cells, and also enhance the migration of leukocytes into tissues [38]. It is essential for several physiological processes, including cell division, growth, and apoptosis. Its potential roles include bone metabolism, bone resorption, and inhibition of bone

formation. The discovery of IL-6 in human gingival tissues and cells increases the likelihood that it plays a role in the molecular processes underlying inflammatory periodontal disease [39]. The presence of MMPs has also been associated with the early detection of peri-implantitis, and the destruction of connective tissue driven by MMPs is an important determinant of the progression of peri-implant lesions, thus MMPs are considered potential biomarkers of peri-implantitis [40,41]. Routine biomarker testing may become a personalized diagnostic tool in clinical practice in the future [42]. The combination of IL-1β, IL-6, and MMP-8 with probing bleeding, gingival index, and probing depth can give a comprehensive explanation of the pathogenesis of peri-implant diseases. The release of proinflammatory cytokines and chemokines, such as IL-1β and IL-6, can lead to bone resorption, which is closely related to disease progression [43]. In the hydrogen-rich water group, the clinical indices improved, and the expression of inflammatory factors significantly decreased. These results suggested that treatment with hydrogen-rich water could inhibit the expression of IL-1, IL-6, and MMP-8 in the peri-implant niche.

The highly permeable junctional epithelium allows various types of mononuclear leukocytes, such as neutrophils, macrophages, lymphocytes, and antigen-presenting cells, to reside in the intercellular gaps. Additionally, the intercellular gap enables leukocytes to enter the sulcus fluid [44]. In this study, we observed significantly fewer inflammatory cells in the treated group compared to those in the control group, indicating that hydrogen-rich water had scavenging and inhibiting effects on inflammatory cells.

The diversity of the oral bacterial community is extensive. Bacteria can colonize an implant within one hour of placement, and a complex biofilm can form within two weeks [45]. Studies have shown that the bacterial biofilm on the implant surface is similar to that on the tooth surface [46]. The microbiome of peri-implantitis is an anaerobic infection caused by a polymicrobial population, and the severity of the disease may not correspond to the microbiome [40]. In addition, peri-implantitis is caused by bacteria, including fungus, Bacteroides, Clostridium, and Treponema, which are also present in periodontitis [47]. Compared with periodontitis, peri-implantitis had a higher infection of actinomycetes aggregators, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Treponema*, and *Tannerella forsythia* [48]. *Staphylococcus aureus* is also associated with peri-implantitis [46]. In this context, a detailed examination of the peri-implant microbiota is necessary to understand the response to treatment. Previous studies have applied a variety of microbial assessment techniques [49]. Metagenomic profiling via 16S rRNA sequencing was used to comprehensively identify various microorganisms using gene and clone library construction techniques. This method can detect the community of species in a given sample and indicate the presence of previously uncultured and unknown bacteria [46]. This high-throughput approach provides abundant information and generates a large amount of data that can be used to identify high levels of microbial diversity [46].

Bacterial infection is the primary cause of peri-implant inflammation [50]. This infection may result from the opportunistic and common proliferation of periodontal pathogenic bacteria on the implant surface. Spirochetes have been associated with peri-implantitis, similar to Gram-negative anaerobic bacteria, particularly *Porphyromonas gingivalis* [51]. *Porphyromonas gingivalis* is a well-known pathogen that invades host cells and leads to the development of peri-implant disease [52]. The lack of periodontal ligament at implants restricts the blood supply to the blood vessels of the implant-supporting tissue, thereby limiting the number of nutrients and immune cells that may be released in response to early bacterial infections. Additionally, the connective tissue fibers surrounding the implant differ from those of natural teeth, as they are oriented horizontally rather than vertically. The anatomical functional structure reduces the physical barrier preventing bacterial invasion of the submucosa, leading to an open wound architecture for the peri-implant tissue [53]. Dental implants are more susceptible to indigenous oral bacteria compared to normal teeth due to variances in basic histology and immunophysiology [54]. In the initial months post-implantation, the biofilm around the implant exhibits minor differences in taxonomic composition, with lower microbial diversity than adjacent teeth [55]. Factors promoting biofilm development also contribute to tissue inflammation initiation and alteration of the peri-implant niche's microenvironment [52]. The accumulation of plaque biofilm on the implant surface leads to peri-implantitis and loss of support [56,57]. In this study, we observed differences in microbiome composition between the treated and control groups. The results revealed that *Porphyromonas* and *Fusobacterium* were the predominant pathogenic bacteria. Additionally, the relative abundance of several pathogenic bacteria in the treated group was lower than that in the control group, suggesting a decrease in pathogenic bacterial abundance under the treatment of hydrogen-rich water. These findings provide important insights for clinical translation. The potential adjunctive use of hydrogen-rich water in standard mechanical therapy for peri-implant disease and during the maintenance phase should be investigated in clinical trials. Despite the challenges posed by poor stability in application, the ease of preparation offers possibilities for patient-performed self-application. Furthermore, the long-term effects on healthy oral tissues also need to be established.

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Conclusions

This study demonstrated that in experimental peri-implantitis, treating the affected area with hydrogen-rich water could decrease the inflammatory response of the peri-implant tissues and result in a significantly lower presence of major pathogenic bacterial genera in the peri-implant crevicular fluid. Therefore, hydrogen-rich water could be considered as a potential treatment for peri-implantitis.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding authors on reasonable request.

Author Contributions

YWZ, ZLZ, and LH designed the research. YWZ performed all of the experiments. YWZ and ZLZ wrote the manuscript. YWZ, SML, GS, CCS, RZ, LH, YYY, JMW, YZ, JX and SYL analyzed the data. CCS and RZ provided technical guidance on the establishment of animal models. YZ and JX administered and supervised the whole research project. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Nanfang Hospital Animal Ethic Committee (NFYY-2020-0351). All experimental procedures adhered to ethical principles and were conducted at the Nanfang Hospital's animal experiment center.

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Conflict of Interest

The authors declare no conflict of interest.

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