

## RESEARCH ARTICLE

# Effects of Hydrogen-Rich Water on Interleukin-1 $\beta$ , Number of Osteoclasts and Osteoblasts in Streptozotocin-induced Diabetic Rats with Orthodontic Tooth Movement

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## Abstract

**BACKGROUND:** Orthodontic tooth movement (OTM) may increase the risk of treatment-related complications for some diabetes mellitus (DM) patients. Hydrogen-rich water (HRW) has been demonstrated in many studies to reduce oxidative stress and cell damage. This study aimed to examine the levels of interleukin (IL)-1 $\beta$ , blood glucose level, body weight, tooth displacement, and population of osteoclasts and osteoblasts in diabetic rats with OTM.

**METHODS:** Thirty rats (*Rattus norvegicus*) were divided into 6 groups: OTM, HRW, DM, DM+OTM, DM+HRW, and DM+OTM+HRW. DM, DM+OTM, DM+HRW, and DM+OTM+HRW groups were induced with streptozotocin (STZ) after 8 weeks of high-fat diet (HFD) and continued being fed HFD for 4 weeks. OTM, DM+OTM, and DM+OTM+HRW groups were placed on an orthodontic device for the orthodontic treatment. HRW, DM+HRW, and DM+OTM+HRW groups were administered HRW via oral

gavage 3 times a day for 4 weeks. At the end of the study, all rats were euthanized and blood samples were collected for IL-1 $\beta$  measurement using enzyme-linked immunosorbent assay (ELISA) kits. Meanwhile, the rats' maxilla was taken to measure tooth movement, and the number of osteoclast and osteoblast were counted.

**RESULTS:** The highest increase of IL-1 $\beta$  was in the DM+OTM group (140.07 $\pm$ 5.14 pg/mL) and the lowest was in the HRW group (92.80 $\pm$ 2.89 pg/mL). The average number of osteoblasts were higher in tension sites, while osteoclasts were higher in pressure sites.

**CONCLUSION:** Consumption of HRW in STZ-induced diabetic rats with OTM can reduce IL-1 $\beta$  levels, reduce tooth mobility, and promote bone remodeling.

**KEYWORDS:** diabetes mellitus, hydrogen rich water, interleukin-1 $\beta$ , orthodontic tooth movement, osteoblast, osteoclast

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## Introduction

Orthodontic treatment in adult patients requires high aesthetic and functional improvement. Some of these patients may have systemic conditions, such as diabetes mellitus (DM). Treatment is a challenge for these DM patients as it can increase the risk of complications during treatment. DM is indicated by high blood glucose levels

and has two primary types. Type 1 DM (T1DM) occurs when the pancreatic beta cells are destroyed by the immune system.(1) Type 2 DM (T2DM) is characterized by insulin intolerance which triggers compensatory mechanisms and induces  $\beta$ -cells to release more insulin.(2)

Inflammation, particularly due to interleukin (IL)-1 $\beta$ , leads to insulin resistance and diabetes.(3,4) Pro-inflammatory cytokines can cause insulin resistance and inhibit insulin signal transduction. Therefore, IL-1 $\beta$  plays

a main role as a biomarker in diabetes and orthodontic tooth movement (OTM). (5) IL-1 $\beta$  is most involved in bone remodeling because it simultaneously suppresses bone growth and increases bone resorption. (6,7) Increased IL-1 $\beta$  levels around teeth receiving OTM may indicate that periodontal cells react to the stresses of orthodontic treatment.

Mediated by released cytokines, OTM induces alveolar bone changes and continues the remodeling process. (8,9) The quality of bone remodeling depends on a regulated inflammatory response, and the activation of cytokines is crucial for the mineralization of tissues. Alveolar bone remodeling through bone resorption on the pressure side and bone apposition on the tension side are two tissue changes connected with OTM. (10) OTM generates mechanical stress that damages tissue and activates inflammatory mediators. As a result, inflammation is an important prerequisite for inducing OTM.

With the presence of abundant hydrogen molecules, hydrogen-rich water (HRW) can prevent oxidative stress and cell damage. Many studies have revealed that HRW can decrease level of blood glucose. (11,12) In dentistry, HRW can reduce gingival oxidative stress due to aging and periodontal disease (13) and reduce the degree of alveolar bone loss (14). Gingival oxidative stress delays alveolar bone loss in mouse models. (13,15) A greater concentration of HRW can reduce interleukin levels and promote wound healing. (16)

Clinical studies showed that consumed HRW affected glucose metabolism and decreased oxidative stress indicators in individuals with T2DM or those with other metabolic syndromes. However, there is no research regarding the effects of HRW on IL-1 $\beta$  and bone tissue remodeling toward orthodontic treatment in patients with DM. Therefore, the researcher wanted to know the effects of HRW on IL-1 $\beta$  of Streptozotocin (STZ)-induced diabetic rats with OTM.

## Methods

A post-test only control group design was conducted, and the protocol of the study has been approved by the Ethics Committee to utilize animals, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia (No. 125/KEPH/VIII/2022).

### Animal Models

Animal models induction and treatments were conducted as shown in Figure 1. The thirty male rats (*Rattus novvergicus*),

aged 10 weeks old and weighed 200-250 g, were acclimated for one week at a constant temperature of 23 $\pm$ 2 $^{\circ}$ C and under a 12/12-hour light/dark cycle. At the end of the acclimatization period, the rats were separated into 6 groups, with 5 rats in each group.

OTM group consisted of healthy rats that were given OTM; HRW group consisted of healthy rats that were given HRW treatment; DM group consisted of diabetes-induced rats that received no OTM and HRW treatment; DM+OTM group consisted of diabetes-induced rats were given OTM, but did not received HRW treatment, DM+HRW group consisted of diabetes-induced rats that were not given OTM, but received HRW treatment; while DM+OTM+HRW group consisted of diabetes-induced rats that were given OTM and HRW treatment.

### Induction of Diabetes

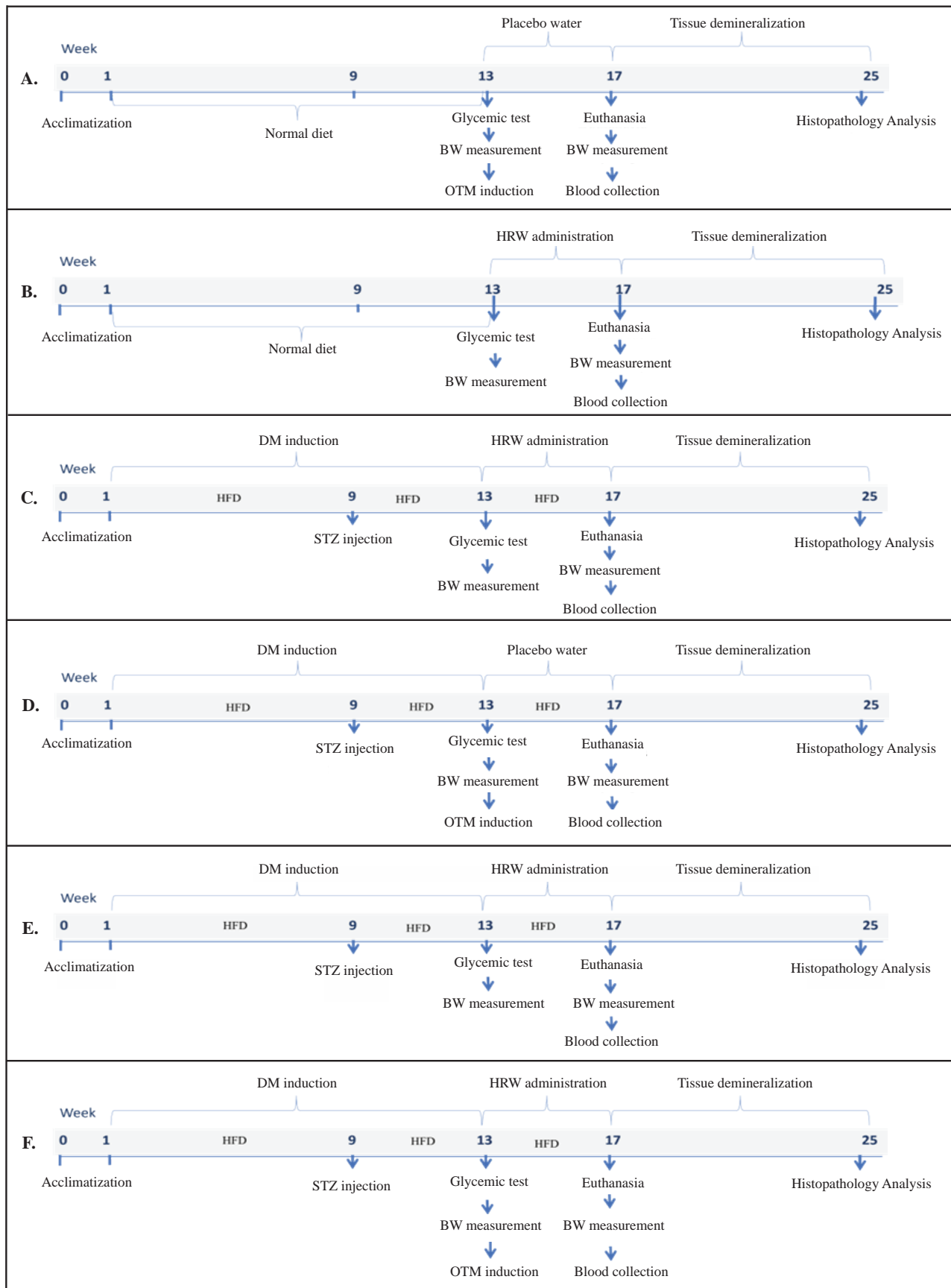
Rats in OTM and HRW groups were given a normal diet and were not induced to be diabetic. While rats in DM, DM+OTM, DM+HRW, and DM+OTM+HRW groups were fed with high-fat diet HFD for 8 weeks, then injected with 30 mg/kg BW STZ intraperitoneally to induce T2DM. The emergence of diabetes was marked by persistent hyperglycemia (17,18), and then HFD was continued for 4 weeks. After that, blood samples were drawn from each rat after 10-hour fasting (19,20), and blood glucose was tested using a digital blood glucometer to verify the condition of DM. Rats were diagnosed with DM when their blood glucose levels reached 200 mg/dL (normal range: 50-135 mg/dL).

### HRW Preparation

HRW was produced by electrolysis in Dr Water electric generator (Altrams, Jakarta, Indonesia) with a concentration of 1,000-1,100 ppm for 5 min at room temperature. Hydrogen concentration was observed with a Hydrometer Tester H<sub>2</sub> YY-400H (Yieryi, Guangdong, China). Groups OTM, DM, and DM+OTM accepted placebo water, and groups HRW, DM+HRW, and DM+OTM+HRW accepted HRW water for 4 weeks. Freshly prepared HRW was fed to the rats via oral gavage in a volume of 5 mL three times a day. To maintain H<sub>2</sub> concentration, the remaining HRW was stored in tightly sealed aluminum bags.

### OTM Treatment

Prior to orthodontic treatment, rats in groups OTM, DM+OTM, and DM+OTM+HRW were injected intramuscularly with 5 mg xylazine + 100 mg ketamine. (21) A 0.2 mm closed coil spring 4 mm long was connected



**Figure 1. Timeline of the experimental treatments conducted in each group. A: OTM, B: HRW, C: DM, D: DM+OTM, E: DM+HRW, F: DM+OTM+HRW.**

to the incisors with a 0.10 mm steel ligature wire, inserted into the interproximal area between the first and second molar teeth using the left maxilla. To prevent harm from the ligature's end, the composite resin was attached after acid etching. The food source of the rats was smashed to protect the orthodontic appliance. When 40 grams of force was applied, stress of the first molar was measured using an orthodontic dental force gauge (Orthdent Laboratory, Buffalo, NY, USA).(22,23)

The rats were sacrificed with an appropriate dose of xylazine plus ketamine at the end of the investigation. After dissecting the jaw, tooth movement was measured from the mesial first molar to the distal third molar and compared with the contralateral side using digital calipers (Mitutoyo Digimatic Caliper, with the precision of 0.01 mm; Mitutoyo Corporation, Sakado, Japan).

### Preparation of IL-1 $\beta$

In this study, blood serum was used for IL-1 $\beta$  testing, and whole blood samples were used for blood glucose testing. Blood samples were taken immediately after the rats were euthanized and blood serum was prepared for IL-1 $\beta$ . The serum was extracted from clot-free plasma or blood without fibrinogen and other clotting agents. Serum, which has less protein than plasma, was utilized more commonly in clinical investigations because certain proteins in plasma or the presence of anticoagulants might affect test results. Every 4 weeks, a total of 7-9% of the rats' overall blood volume or 1% of their entire body weight was collected. Rats were housed in a small container and warmed with an infrared lamp to stimulate the tail's blood flow. After that, a restraint system was used to hold the rat while collecting the blood from the tail.(24)

To collect the blood, alcohol was used to disinfect the tail, and a sterilized scalpel was used to slightly cut the tip of the tail. A few drops of blood were drawn by holding the tail over the blood collection tube, and simultaneously the tail was gently massaged distally to test blood glucose levels. When the bleeding did not stop on its own, pressure was applied for 2 to 3 min.(21,24) The blood sample was kept at 37°C for 1 hour. The tube was flicked several times to dislodge the blood clot. The tube was transferred to 4°C and incubated for 2 hours. Then the blood was centrifuged at 10,000 g for 10 min at 4°C and discarded the platelet cells from the serum. The serum was recentrifuged for 10 min. Confirm that there were no platelet cells in the extracted serum.(24) IL-1 $\beta$  was assayed using the enzyme-linked immunosorbent assay technique (ELISA) methods using Rat ELISA IL-1 $\beta$  set (Elabscience, Houston, TX, USA).

### Histopathology Preparations

Epithelial tissues and muscular components around the maxilla of the euthanized rats were immediately taken. The tissue samples were demineralized in EDTA for 8 weeks after being fixed in a 10% formaldehyde buffer solution for 3 days (72 hours), after that, the paraffinized tissues were microtomed cross-sectionally in 5  $\mu$ m thick. The samples were measured from several roots of the mobilized tooth. The osteoblast population of the periodontal ligament was counted on the tension side, while the osteoclast population was counted on the pressure side. The distal side was used to measure osteoblasts because of the reaction in the tension region. Multinucleated osteoclasts were counted on the mesial because of the reaction on the pressure side. Staining was done with hematoxylin-eosin (HE).(25)

## Results

### IL-1 $\beta$ Levels

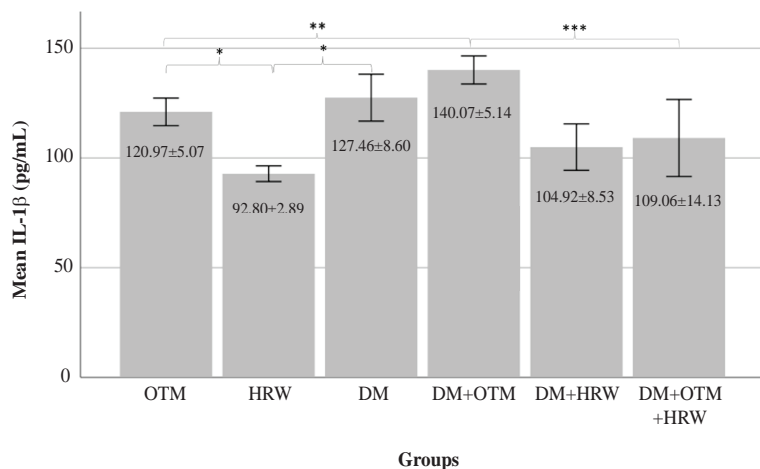
Figure 2 showed that the highest increase in IL-1 $\beta$  by ELISA quantification was found in the DM+OTM group (140.07 $\pm$ 5.14 pg/mL) and the lowest was in the HRW group (92.80 $\pm$ 2.89 pg/mL). The data showed that DM+HRW group had lower IL-1 $\beta$  level (104.92 $\pm$ 8.53 pg/mL) compared to the DM group (127.46 $\pm$ 8.60 pg/mL), which indicated a decrease in IL-1 $\beta$  after HRW administration. The OTM group had a higher IL-1 $\beta$  level (120.97 $\pm$ 5.07pg/mL) than the DM+OTM+HRW group (109.6 $\pm$ 14.13 pg/mL), which meant that eventhough rats undergone OTM and had DM, but after HRW administration, it would had lower IL-1 $\beta$  level than rats that undergone OTM treatment only.

### Orthodontic Tooth Movement

Table 1 displayed the impact of tooth displacement. The deviation between the moving side and the contralateral side was calculated to measure the level of movement in each group that underwent OTM. Comparing the moved side and the contralateral side, the DM+OTM group had statistically significant higher tooth movement (7.07 $\pm$ 0.06 mm) on the moved side compared to the contralateral. No significant difference was found in the measurement results of OTM and DM+OTM+HRW groups.

### Blood Glucose Levels

Table 2 demonstrated that diabetic rats had impaired glucose tolerance as seen by the fact that blood glucose levels significantly rose after HFD treatment and STZ injection, indicating that glucose tolerance has been reduced. After



**Figure 2. IL-1β levels 4 weeks after OTM treatment and HRW administration.** The data was displayed as mean±standard deviation (SD). If analysis using One-way ANOVA showed  $p < 0.05$ , then HRW had an effect on IL-1β of DM rats. The Tukey post-test was then used for the follow up test to compare the values. \*HRW group had significant decrease of IL-1β level compared to DM and OTM groups ( $p = 0.000$ ). \*\*DM+OTM group had significant increase of IL-1β level compared to OTM group ( $p = 0.014$ ). \*\*\*DM+OTM group had significant increase of IL-1β level compared to DM+OTM+HRW group ( $p = 0.000$ ).

4 weeks post STZ injection, the blood glucose levels in HRW, DM+HRW, and DM+OTM+HRW groups were significantly decreased compared to the OTM, DM, and DM+OTM groups ( $p < 0.05$ ). Blood glucose levels appeared to be strongly influenced by food intake and consumption of HRW.

**Body Weights**

All group animals gained similar amounts of weight during the first phase of the experiment. After 4 weeks the DM group had significant changes due to the STZ-induction. However, the groups that received HRW administration (HRW, DM+HRW, DM+OTM+HRW groups) did not show a significant decrease in body weight (Figure 3).

**Histopathology Analysis**

Figure 4 showed the histopathology analysis results of average osteoclast and osteoblast cell numbers in all groups. The average number of osteoblasts in tension sites was significantly higher than in the pressure sites, while the average number of osteoclasts was seen significantly higher in the pressure sites. On the pressure side, the highest average number of osteoclasts was shown in the DM+OTM group ( $13.60 \pm 1.14$ ), and the lowest number was found in OTM group ( $2.80 \pm 0.84$ ). Meanwhile, on the tension side, the highest average number of osteoblasts was seen in

the DM+OTM+HRW group ( $21.00 \pm 1.41$ ) and the lowest number was found in the DM+OTM group ( $8.60 \pm 1.14$ ) (Table 3).

**Discussion**

In this study, the highest IL-1β level was found in the DM+OTM group. This indicates that there is a significant increase in IL-1β levels in diabetic-induced rats that were treated with OTM. Normal IL-1β levels in the serum of experimental rats by ELISA analysis were  $97.36 \pm 63.84$  pg/mL. (26) Inflammation, especially due to IL-1β signaling leads to glucose intolerance progression and DM. (27,28) The administration of HRW reduced IL-1β levels, as shown in HRW, DM+HRW, and DM+OTM+HRW groups. This finding is supported by report which indicated that HRW significantly suppresses inflammatory responses, oxidative stress levels, and bacterial growth processes so that it can prevent, manage, and maintain periodontal support tissues and inflammatory damage. (29) HRW can decrease the level of IL-1β. (30)

This study used a 40 g of force to move the rats' teeth. The greatest tooth movement was seen significant in OTM+DM, followed by OTM and OTM+DM+HRW which did not show significant difference. Significant differences

**Table 1. Tooth displacement measurement results.**

Group	Moved Side (mm)	Contralateral (mm)	p-value
OTM	6.94±0.15	6.59±0.26	0.113
DM+OTM	7.07±0.06	6.60±0.15	0.039*
DM+OTM+HRW	6.95±0.24	6.67±0.25	0.127

\*DM+OTM group has statistically significantly higher movement compared to its contralateral. Tested with unpaired T-test, considered significant if  $p < 0.05$ .

**Table 2. Blood glucose level at 0 and 4 weeks after DM-induction.**

Group	Week-0	Week-4	$\Delta$	<i>p</i> -value
OTM	107.40±18.24	108.60±16.94	1.2±6.76	0.712
HRW	104.00±12.71	95.40±5.46	8.6±10.31	0.136
DM	271.20±23.85	270.80±20.99	0.4±30.12	0.978
DM+OTM	238.40±22.57	293.00±25.50	54.6±29.05	0.014*
DM+HRW	274.00±17.65	194.80±23.44	79.2±31.87	0.005**
DM+OTM+HRW	245.00±19.61	199.80±26.70	45.2±18.03	0.005**

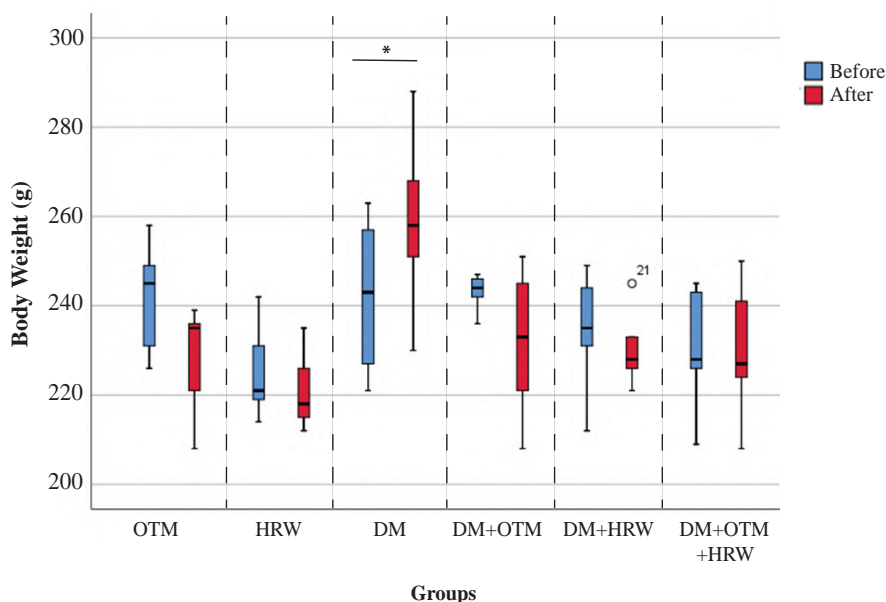
Blood glucose level was presented in mg/dL.  $\Delta$ : difference before and after intervention, and the *p*-value was tested with T-test, with significance of  $p < 0.05$ . \*DM+OTM group had significant increased blood glucose levels between 0 week and after 4 weeks of HRW administration. \*\*DM+HRW and DM+OTM+HRW groups had significant decreased blood glucose levels between 0 week and after 4 weeks of HRW administration.

were found among diabetic and non-diabetic groups with tooth mobility shown by the DM group, this finding is supported by the findings of Braga's study, which showed increased tooth mobility in the presence of diabetes.(31) OTM exacerbates the inflammatory response of periodontal tissues, leading to extended damage to the periodontium. (32) This result also appears in this study which showed that DM+OTM increased tooth mobility.

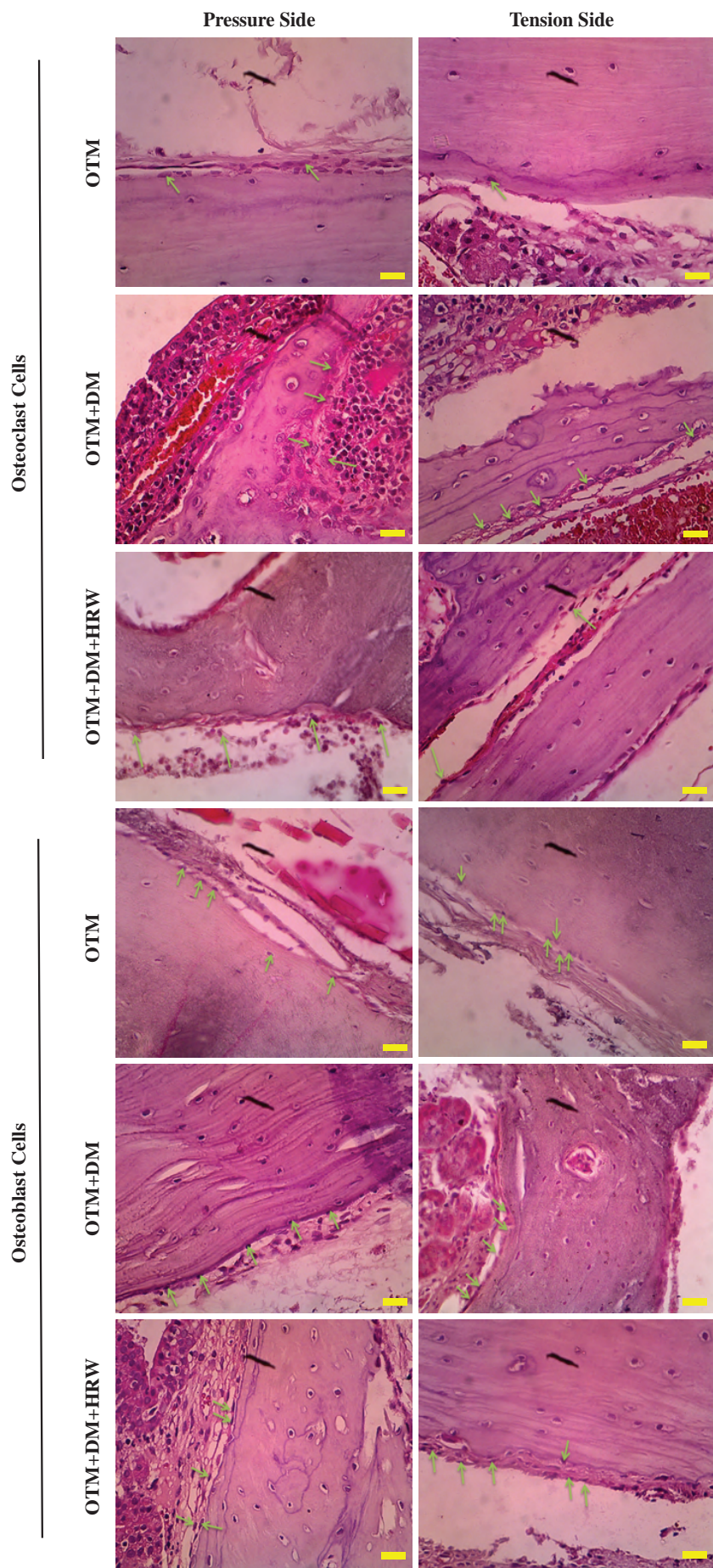
Many studies on HRW show that it can reduce blood glucose levels in DM patients.(11,12,33) HRW can protect cells from oxidative stress so that it can delay the onset of insulin resistance and the occurrence of T2DM.(11) Oxidative stress refers to the condition where there is an accumulation of free radical molecules. Oxidative damage arises as a manifestation of DM.(34) HRW serves as a crucial defense mechanism against oxidation and plays a vital role in safeguarding cells against harm caused by free

radicals. Table 2 and Figure 3 of this study results show that consuming HRW for 4 weeks among diabetic-induced rats can significantly reduce blood glucose levels, but not significant weight loss. This result is in line with there was no significant difference in body weight between the control and experimental groups.(14) Different from other findings which claimed that HRW significantly suppressed body weight after 18 weeks of consuming HRW.(12)

OTM treatment in diabetic rats can promote bone resorption and periodontal ligament injury.(35) In healthy animals subjected to sufficient orthodontic pressure, bone resorption, and apposition occurred without significant injury. However, the number of osteoclasts was elevated in diabetic rats, resulting in more bone resorption. The increased level of apoptosis in periodontal ligament osteoclasts and fibroblasts, which accelerates periodontal destruction and impairs bone healing, is an effect of



**Figure 3. Body weights comparison before (0 week) and after (4 weeks) HRW administration.** \*DM group showed significant weight gain. All groups comparison was tested using T-test, with significance of  $p < 0.05$ .



**Figure 4. Effect of HRW on the osteoclast and osteoblast cell numbers.** Epithelial tissues of rats' jaw was prepared in 5  $\mu$ m. Green arrows show osteoclast and osteoblast cells. Yellow bar: 200 $\mu$ m.

**Table 3. Osteoclast and osteoblast cell numbers on pressure and tension side.**

Group	Osteoclast	Osteoblast
<b>Pressure</b>		
OTM	2.80±0.84	11.60±1.14
DM+OTM	13.60±1.14*	6.20±0.84
DM+OTM+HRW	3.80±0.84	18.00±1.00
<b>Tension</b>		
OTM	2.00±0.71	14.60±1.14
DM+OTM	9.00±1.22	8.60±1.14
DM+OTM+HRW	2.00±0.71	21.00±1.41**

\*In DM+OTM group, pressure vs. tension ( $p<0.05$ ) has a statistically significant higher osteoclast population in the pressure site than tension site. \*\*In DM+OTM+HRW group, pressure vs. tension ( $p<0.05$ ) has significantly higher osteoblast in the tension site than pressure site.

hyperglycemia, which also causes metabolic changes that result in tissue degradation through reduced neutrophil activity. Table 3 shows that the mean value of osteoclast count in the tension site significantly decreases in the DM+OTM+HRW group compared to the DM+OTM group and the mean value of osteoblast number in the tension site increased significantly in the DM+OTM+HRW group. This finding shows that the treatment with HRW among diabetic-induced rats with OTM could reduce the population of osteoclasts and could increase the number of osteoblasts on the tension side compared to those without HRW, so can be concluded that HRW can help to maintain the balance of tooth remodeling on OTM. It was also reported that HRW can inhibit osteoclastogenesis and prevent excessive bone resorption.

The prolonged inflammatory response that occurs in hyperglycemia slows the healing process, this stimulation encourages bone resorption and obstructs bone growth. In diabetic-induced *Rattus norvegicus*, HRW consumption can accelerate bone remodeling.(27) HRW counteracted the negative effects of aging on osteoclast development, by lowering oxidative damage, resulting in a decreased rate of alveolar bone loss in the HRW-treated group than the control group. The administration of HRW can reduce gingival oxidative stress and stop the loss of alveolar bone in a mouse model.(13) Since this study analyzed the effects of HRW on IL-1 $\beta$  levels, blood sugar levels, body weight, osteoblasts and osteoclasts in DM induction rats, it is also important to investigate the role of HRW in other metabolic diseases. Further research is needed to confirm the effects of HRW with other biomarkers, extend the period of HRW administration, and increase the sample size.

## Conclusion

Consumption of HRW in STZ-induced diabetic rats with OTM can reduce IL-1 $\beta$  levels and promote bone remodeling by increasing osteoblast on the tension side and decreasing osteoclast on the pressure side. At the same time, HRW can also decrease body weight although not significantly.

## Authors Contribution

LH, AMH, and F were involved in the conception and planning of the research. LH and AMH performed the methodology, interpreted the results, and designed the figures. LH performed the data acquisition/collection, calculated the experimental data, and performed the analysis. All authors took part in manuscript preparation and gave critical revisions to the manuscript.

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