Iraqi Journal of Veterinary Sciences

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Effects of local rosuvastatin/hyaluronan hydrogel on post-orthodontic relapse reduction in rabbits: Histological and immunohistochemical study

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Article information

Article history:
Received 20 July, 2024
Accepted 01 November, 2024
Published online 01 January, 2025

Keywords:
Glycosil
Immunohistochemistry
Orthodontic
Relapse
Rosuvastatin

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Abstract

One of the most novel approaches to reducing post-orthodontic relapse is the local delivery of osteogenic agents by drug carriers. This study evaluated the effect of a local injection of rosuvastatin (RSV) carried by hyaluronan hydrogel (HAH) on alveolar bone regeneration. On the first day of retention after orthodontic movement of the rabbit's lower incisors, 15 albino rabbits were injected locally with 200 µl of phosphate-buffered saline, and 15 rabbits were injected with 200 µl of RSV/HAH. Histological analysis of alveolar bone remodeling by measuring osteoblast, osteoclast, and blood vessel counts, as immunohistochemical analysis for Bone Alkaline Phosphatase (BAP) and Tartrate Resistant Acid Phosphatase 5b (TRAP 5b) expressions at the injection site at 0, 10, and 21-day intervals at coronal and apical levels was done. Statistical analysis done by SPSS using $P \leq 0.05$ was considered significant. The RSV/HAH group showed a significant increase in osteoblast count and BAP expression on day 0 (score 3+) and day 10 (score 2+), a significant reduction in osteoclast counts, and TRAP 5b expression on day 0 (score 1+) and day 10 (score 2+) with a significant increase in blood vessel count on day 10 both coronally and apically. In conclusion, local injection of RSV/HAH has promising effects on alveolar bone regeneration by increasing BAP expression, osteoblast, and blood vessel counts and reducing TRAP 5 b expression and osteoclast count.

DOI: 10.33899/ijvs.2024.152132.3796,

Introduction

Relapse is the ability of the teeth to return to their original position after orthodontic treatment. The etiology is unknown because post-orthodontic relapse movement is a complex and multifactorial process. One of the most important aspects of relapse processes is alveolar bone remodeling. Mature bone, which is more stable and resistant to resorption, must replace immature bone following orthodontic movement (1). When taken systemically, pharmacological medications can effectively prevent relapse (2), but doing so can adversely affect the entire skeleton, such as an increase in bone mineral density (3). Treatment for periodontal bone loss may be most effective when applied locally. Furthermore, it is still challenging to develop a suitable method of delivering the medication with the best possible bioavailability (4,5). Statins, such as atorvastatin, lovastatin, pravastatin, and rosuvastatin, were primarily created to lower plasma cholesterol levels and stop cholesterol from being produced (6). A novel, third-generation, synthetic, hydrophilic, and extremely effective statin is rosuvastatin (RSV). RSV exhibits pleiotropic effects, including vascularization enhancement, bone stimulation, and inflammation reduction. Compared to atorvastatin and simvastatin, it is more effective (7). When applied locally, RSV has strong effects that boost BMP-2 expression and alkaline phosphatase activity, encouraging osteoblastic growth and preventing osteoclastic bone resorption (8). Based on a systematic assessment of animal research evaluating the impact of statins on relapse following

orthodontic treatment, Afshari *et al.* (6) found that the effect of statins on relapse remains debatable. While several creative strategies affect bone remodeling and limit osteoclastogenesis to decrease post-orthodontic relapse, the most innovative strategy is the local delivery of osteogenic drugs transported by drug carriers (5,9). Hydrogels are three-dimensional, crosslinked scaffolds that are water-swollen. They are biocompatible and may incorporate small particles, drugs, and cells (10). Hyaluronic acid (H.A.) is extensively used to prepare biomaterials for tissue engineering (11). It is a high-molecular-weight glycosaminoglycan (GAG), a polymer with intriguing properties that occurs naturally. Because of its biocompatibility, biodegradability, adaptability, and special qualities that mimic living tissues, H.A. has recently gained popularity as a biopolymer as a starting material for hydrogel designs. These qualities make the hydrogel attractive carriers for the localized and targeted delivery of different drugs (12,13). Modified thiolated hyaluronan hydrogel is an injectable matrix developed to repair bone and articular cartilage abnormalities. This system is usually in solution form before delivery, but it can undergo gelation after injection under physiological conditions. It can also be injected in liquid form, avoiding surgical implantation into tissue with minimal invasiveness (14). Local injection of RSV loaded by thiolated hyaluronan hydrogel can reduce post-orthodontic relapse clinically (15).

However, there is no available data about the effect of local administration of this formula on alveolar bone remodeling. The aim of this study was to evaluate the histological effects of RSV/thiolated hyaluronan hydrogel on the bone after local injection in orthodontically treated rabbits. Also, evaluate its immunohistochemical (IHC) effects by estimation of Bone Alkaline Phosphatase (BAP) and Tartrate Resistant Acid Phosphatase 5b (TRAP 5b) expression upon immunohistochemistry. Therefore, this injectable hydrogel-drug combination could stimulate bone tissue regeneration in a way that may be helpful in post-orthodontic relapse reduction.

Materials and methods

Ethical approval

The Institutional Animal Care and Use Committee, University of Mosul, College of Veterinary Medicine, Ministry of Higher Education and Scientific Research, Iraq, approved all guidelines and experimental protocols for this study, with the approved reference number U.M.VET.2023.094.

RSV/Thiolated hyaluronan hydrogel formula preparation

Rosuvastatin calcium (Awamedica Erbil, Iraq) was loaded into thiolated hyaluronan hydrogel (HAH) named (Glycosil®) (Advanced BioMatrix, USA). (1 mg) was the dose of local application of RSV on bone in the rabbit model (8). According to manufacturer instructions, Glycosil® came at room temperature for 1 hour for loading 1mg/200 µl RSV into each rabbit. 1 mL of degassed, deionized water (D.I.) was added to each vial of Glycosil®. After the addition of water, vortex each vial for seconds. This solution was added to the RSV suspension. Besides, 0.5 mL of D.I. water was added to the thiol-reactive crosslinker vial (Extra Link-Lite®) and was agitated until complete dissolution. Later, 0.25 mL of crosslinker was poured into the Glycosyl-drug mixture. A 3D hydrogel was created when Extra Link-Lite was added to Glycosil® in a 1:4 volume ratio (0.25 mL Extra Link-Lite to 1.0 mL Glycosil®). This was stated in the direction of the use of Glycosil®.

The prepared injectable formula (RSV/HAH) was used for local injections after orthodontic tooth movement. In our previous research, several in vitro investigations of the hydrogel before and after RSV loading were performed to evaluate the properties of this novel formula by evaluating FTIR, ^{1}H-NMR, SEM, gelation behavior, injectability, swelling behavior, and release kinetics of RSV with an evaluation of its clinical effect in vivo (15).

Animal samples and experimental groups

For evaluation of the ability of RSV/HAH on alveolar bone remodeling after orthodontic tooth movement, adult male albino rabbits with an average age of 6.5 months and an average weight of approximately 1200 g were used. They were kept in an animal house set up specifically for this experiment, with 12 hours of light and dark and the same humidity, temperature, and ventilation. Throughout the experiment, they had an unrestricted approach to tap water and a sufficient, steady wheat, corn, and green vegetables diet. The formula that was utilized to establish the sample size was as follows; [n = (z r/D) 2]. (95% confidence); n = required sample size; z (constant) = 1.96 units; r = precision = 0.2 units (2,16). The final sample size was calculated after adjustment, and the final sample size = (z)\ group. According to this, the estimated sample size for each subgroup was five animals. A total sample of 30 rabbits was randomly divided into two groups. Group I (control negative): 15 rabbits received orthodontic appliances and were injected locally with a 200µl phosphate-buffered saline (PBS) solution. Group II (RSV/HAH): 15 rabbits received orthodontic appliances and were injected locally with 200 µl injectable thiolated hyaluronan hydrogel drug carrier loaded with (1mg) RSV. For histological evaluation, each group was subdivided into three subgroups of 5 rabbits according to the time of sacrifice (0, 10, and 21 days) after removing the orthodontic appliance.

Orthodontic treatment

Modified orthodontic appliances (Figure 1) that exerted a lateral reciprocal force of about 50 g were mounted into rabbit incisor teeth (16,17). Before appliance insertion, general anesthesia was given to the rabbits by intramuscular

injections of (50 mg/kg body weight) ketamine hydrochloride (SIR ALDAWA CO, Baghdad, Iraq) and (10 mg/kg body weight) xylazine hydrochloride (Interchemie-Holand) (18). Fixation of orthodontic appliances was done by Ortho Bite Light Curing Composite (FGM, Brazil). The orthodontic force was used to move the lower incisors of rabbits distally for two weeks (18). After these two weeks, the open springs become inactive and are coated with cold-cured acrylic resin (Major, Italy) as a retainer for three weeks.

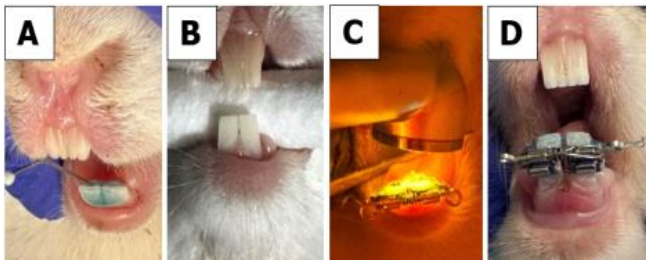

Figure 1: Steps of appliance insertion: A: Acid etching. B: Etched enamel surface. C: Appliance fixation with light curing. D: Appliance after curing.

Local injection

On the first day of the retention period, and after the general anesthesia, local submucosal injection by using a disposable insulin syringe with a 23-gauge needle was administered with the specified solution for each group. The total amount injected was 200 µl. 100 µl was injected into the labial aspect and 100 µl into the lingual aspect of the vestibular mucosa (17,18). All injections were volumetrically equal and were administered once. The needle was inserted through the attached gingiva, in the muco-gingival junction, close and parallel to the mesial surface of the experimental side (left mandibular incisor).

Histological evaluation

After 0, 10, and 21 days of orthodontic appliance removal, rabbits were euthanized for histological analysis. The working field (W.F.) was the mesial side of the lower left central incisors, representing the pressure side during relapse tooth movement, and it was the site of local injection for enhancing bone formation. Histological analyses were conducted at two specified levels (coronal and apical). These regions were determined by drawing parallel lines perpendicular to the long axis of the root of the central incisors and mandibular symphysis, one at the alveolar crest level and the other line about 6000 µm apical to the first line (18), then splitting the interval between the two lines into two equal segments of 3000 µm for each (Figure 2). Six random fields at each level were analyzed under a light microscope (OPTICA Microscopes, Italy) at 400X magnification, and the average value of the readings was recorded. The histological sections were photographed using an OMAX digital camera with image processing software (OMAX 9MP, China). Quantitative analysis of the working field at 0, 10, and 21-day intervals at coronal and apical levels was done to evaluate the effect of the injected material on the alveolar bone at the site of injection regarding the following parameters: osteoblast count, osteoclast count, and blood vessel count.

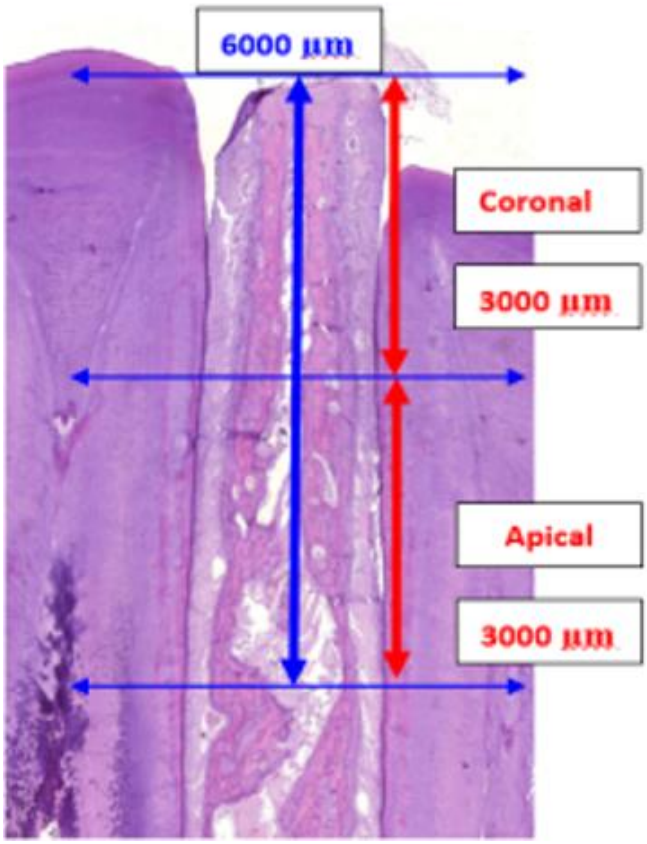

Figure 2: Micrograph demonstrating apical and coronal histological levels of alveolar bone near the left central incisor tooth. The image was taken using 3DHISTECH Panoramic Slide Scanning (Budapest, Hungary) with the software program 3DHISTECH's Slide Center Case-Viewer ver.2.1,3 (Hungary). Staining H&E. Magnification 50X.

Immunohistochemistry protocol

The paraffin sections were dewaxed using xylene, rehydrated, and then brought to deionized water. Antigen retrieval was done using 5% citric acid and washing with PBS. The slides were placed in 3% hydrogen peroxide to block endogenous enzymes and washed with PBS (19-21). A normal goat-blocking buffer was added, and the incubation was at 37°C for 30 min. The primary antibodies in this research include Bone Alkaline Phosphatase Polyclonal Antibody (Elabscience, USA) and Tartrate Resistant Acid Phosphatase 5b Polyclonal Antibody (BioVendor, Brno, Czech Republic). Each primary antibody

was added to its specific slide with the proper dilution ratio (1:50) and kept at 4°C overnight, then rewarmed at 37°C for 30 min. Then, the slides were washed with PBS and dried. Polyperoxidase-anti-Mouse/Rabbit IgG was added to the slides and incubated at 37°C for 20 min, then washed with PBS. 1 drop of DAB concentrate was added into each 1 mL of DAB substrate and thoroughly mixed, then washed with deionized water. The sections were counterstained using hematoxylin, washed with PBS, and then with tap water (22-24). Dehydration was done by ethanol and cleared by xylene. One drop of DPX was applied on each slide. Then, the cover slide was applied (19).

Interpretation of immunohistochemistry

The expression of BAP and TRAP5b was done on a semiquantitative scale that determined the area of immunoreactions at (100X), then measured the intensity of immunoreactions in the working field under a light microscope at a high-power magnification (400 X) and provided grades from zero to three according to the intensity of the immunoreaction, as follows: 0 = no reaction (no staining), 1 = mild immunoreaction (<10% of cells staining), 2 = moderate immunoreaction (10%-50% of cells staining), and 3 = strong immunoreaction (>50% of cells staining) (25). Immunohistochemistry slide analyses were done the same as histological analyses at the apical and coronal levels of the alveolar bone mesial to the root of the lower left central incisor tooth. All histological and immunohistochemical sections were blindly evaluated by two specialized histopathologists, and then the results were statistically analyzed.

Statistical analysis

Statistical analysis of the data for the histological results on the coronal and apical levels at 0, 10, and 21-time intervals between the two groups was performed using SPSS Statistics, Version 26 (IBM Corporation, USA) by an independent sample t-test after checking the normality for all variables using the Shapiro-Wilk test of normality. $P \leq 0.05$ was considered significant. The non-parametric data of the BAP and TRAP 5b biomarker scores were analyzed as median and IQR (Inter-Quartile-Range) by a Mann-Whitney test between groups with a significant level set at $P \leq 0.05$.

Results

Histological effect of injectable materials on alveolar bone remodeling

The histological findings of this study for evaluating the effect of local injection of the RSV/HAH compared with the control group (PBS) on bone remodeling regarding osteoblast, osteoclast, and blood vessel count were as the following (Table 1).

Table 1: Histological findings of osteoblast, osteoclast, and blood vessel count between the control (I) and RSV/HAH (II) groups at coronal and apical levels on 0, 10, and 21 days post-appliance removal

Variable	Region	Group	Day 0		Day 10		Day 21	
			Mean±SD	P value	Mean±SD	P value	Mean±SD	P value
Osteoblast	Coronal	I	15.20±0.200	0.000	9.20±0.209	0.000	8.12±0.867	0.175
		II	28.16±0.040		17.80±0.079		9.00±1.000	
	Apical	I	13.80±0.758	0.000	9.10±0.100	0.000	7.49±0.500	0.976
		II	26.20±0.200		13.99±0.507		7.50±0.500	
Osteoclast	Coronal	I	1.91±0.883	0.000	2.80±0.200	0.000	2.01±0.188	0.405
		II	1.10±0.100		2.10±0.100		1.92±0.130	
	Apical	I	1.61±0.192	0.000	2.50±0.192	0.000	1.80±0.197	0.079
		II	0.82±0.083		1.80±0.095		1.60±0.092	
Blood vessels	Coronal	I	2.00±0.202	0.158	2.30±0.097	0.000	2.20±0.100	0.717
		II	2.20±0.200		3.30±0.097		2.22±0.102	
	Apical	I	1.81±0.201	0.397	2.00±0.105	0.000	2.00±0.100	0.976
		II	1.90±0.100		2.80±0.100		2.00±0.102	

Data expressed as Median±SD. N=5. Significant difference existed at $P \leq 0.05$. (I)= control group. (II) = RSV/HAH group.

Osteoblast count results

In the coronal and apical parts of the bone, osteoblasts (Figure 3) were significantly increased in the RSV/HAH group compared to the control group on day 0 and day 10. At the same time, a non-significant difference was found on day 21. Osteoblast count in both groups was the highest on day 0 and decreased progressively with time.

Osteoclast count results

In the coronal and apical parts of the bone, osteoclasts (Figure 4) were significantly decreased in the RSV/HAH group compared to the control group on day 0 and day 10. At the same time, a non-significant difference was found on day 21. Osteoclast count in both groups was the lowest on day 0, increased on day 10, then reduced on day 21.

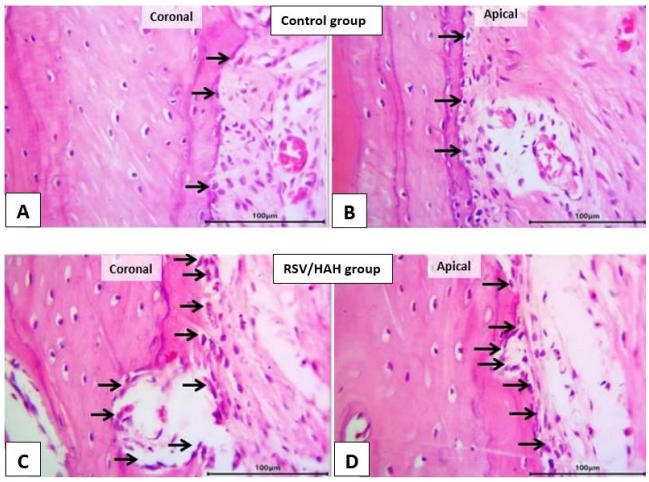

Figure 3: Histological sections of rabbit's lower left central incisor on day 10 post-orthodontic appliance removal showing the osteoblasts count (arrows). A&B: Control group. C&D: The RSV/HAH group is showing an abundance of osteoblasts. Left panel: Coronal region. Right panel: Apical region. Scale-bar=100µm. Staining H&E. Magnification 400X.

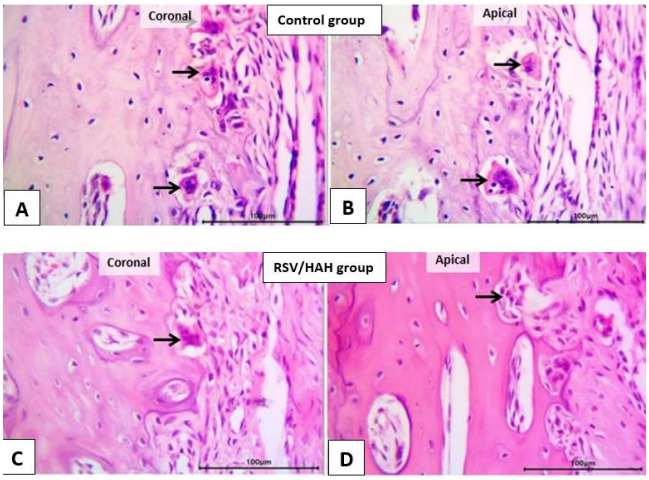

Figure 4: Histological sections of rabbit's lower left central incisor on day 10 post-orthodontic appliance removal showing the osteoclasts count (arrows). A&B: Control group. C&D: RSV/HAH group. Left panel: Coronal region. Right panel: Apical region. Scale-bar=100µm. Staining H&E. Magnification 400X.

Blood vessel count results

In the coronal and apical parts of the bone, blood vessels (Figure 5) were significantly increased in the RSV/HAH group compared to the control group on day 10. At the same time, a non-significant difference was found on day 0 and day 21.

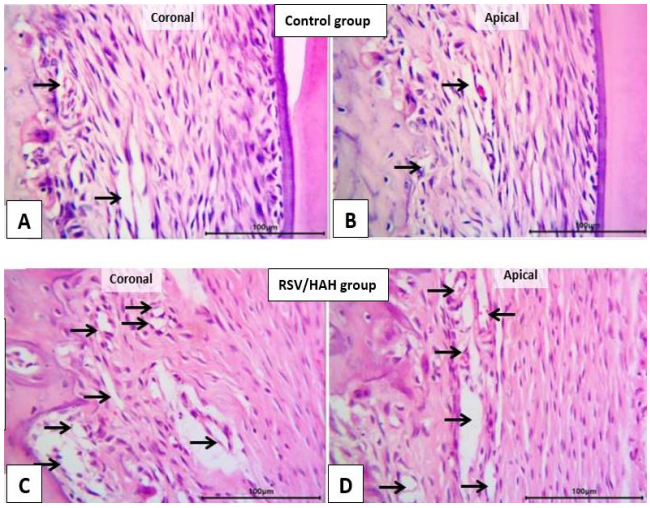

Figure 5: Histological sections of rabbit's lower left central incisor on day 10 post-orthodontic appliance removal showing the blood vessels count (arrows). A&B: Control group. C&D: RSV/HAH group. Left panel: Coronal region. Right panel: Apical region. Scale-bar=100µm. Staining H&E. Magnification 400X.

Immunohistochemistry results

The immunohistochemical stain of the bone section at the W.F. revealed a positive reaction for BAP and TRAP 5b in both groups, coronally and apically, with different immunoreactions. The immunohistochemical analysis showed a significant increase in the BAP expression on day 0 from moderate expression (score 2+) for the control group to strong expression (score 3+) for the RSV/HAH group, both coronally and apically. Also, a significant increase in the BAP expression was found on day 10 from weak expression (score 1+) for the control group to moderate expression (score 2+) for the RSV/HAH group both coronally and apically. Furthermore, RSV/HAH and control groups showed weak BAP expression (score 1+) both coronally and apically on day 21 (Table 2) (Figures 6 and 7).

Immunohistochemical analysis of TRAP 5b on day 0 showed a significant decrease in the TRAP 5b expression from moderate (score 2+) for the control group to weak (score 1+) for the RSV/HAH group, both coronally and apically. Also, on day 10, there was a significant decrease in the TRAP 5b expression from strong (score 3+) for the control group to moderate (score 2+) for the RSV/HAH group, both coronally and apically. Finally, on day 21, RSV/HAH and control groups showed moderate expression (score 2+) of TRAP 5b stain both coronally and apically (Table 3) (Figures 8 and 9).

Table 2: Scores of Immunohistochemistry expression of the BAP of the control and RSV/HAH groups on 0, 10, and 21 days of the study periods

Variable	Region	Group	Day 0		Day 10		Day 21	
			Median±IQR	P value	Median±IQR	P value	Median±IQR	P value
BAP	Coronal	I	2±0.0	0.008	1±0.5	0.032	1±0.5	0.690
		II	3±0.0		2±0.5		1±0.0	
	Apical	I	2±0.5	0.032	1±0.5	0.032	1±0.5	0.421
		II	3±0.5		2±0.0		1±0.5	

Data expressed as Median±SD. N=5. Significant difference existed at P≤0.05. (I)= control group. (II) = RSV/HAH group.

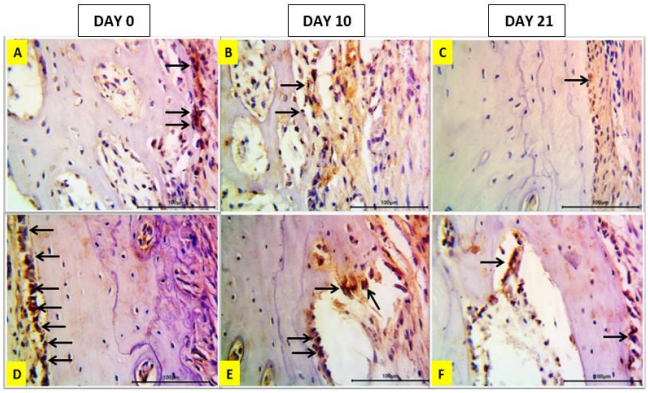

Figure 6: Immunohistochemistry expression of the BAP in the lower left central incisor at the coronal level of rabbit from control (upper panel) and RSV/HAH (lower panel) groups on days 0, 10, and 21 post-orthodontic appliance removal reveals a weak positive reaction (Score 1+) in the control group on day 10 (B); and in the control and RSV/HAH groups on day 21 (C, F). A moderate positive reaction (Score 2+) in the control group on day 0 (A) and in the RSV/HAH group on day 10 (E). A strong positive reaction (Score 3+) in the RSV/HAH group on day 0 (D). (The positive reaction is the dark brown color of osteoblasts (arrows). 400X.

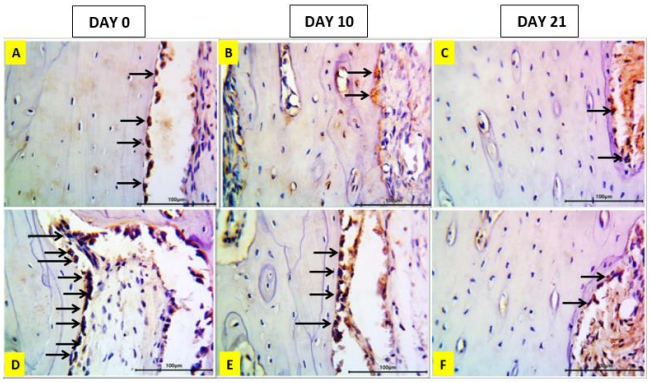

Figure 7: Immunohistochemistry expression of the BAP in the lower left central incisor at the apical level of rabbit from the control (upper panel) and RSV/HAH (lower panel) groups on days 0, 10, and 21 post-orthodontic appliance removal reveals a weak positive reaction (Score 1+) in the control group on day 10 (B); and in the control and RSV/HAH groups on day 21 (C, F). A moderate positive reaction (Score 2+) in the control group on day 0 (A) and in the RSV/HAH group on day 10 (E). A strong positive reaction (Score 3+) in the RSV/HAH group on day 0 (D). (The positive reaction is the dark brown color of osteoblasts (arrows). 400X.

Table 3: Scores of immunohistochemistry expression of the TRAP 5b of the control and RSV/HAH groups on 0, 10, and 21 days of the study periods

Variable	Region	Group	Day 0		Day 10		Day 21	
			Median±IQR	P value	Median±IQR	P value	Median±IQR	P value
TRAP 5b	Coronal	I	2±0.5	0.032	3±0.0	0.008	2±0.5	1.000
		II	1±0.5		2±0.0		2±0.5	
	Apical	I	2±0.0	0.032	3±0.5	0.032	2±0.5	1.000
		II	2±0.5		2±0.5		2±0.5	

Data expressed as Median±IQR. N=5. Significant difference existed at P≤0.05. (I)= control group. (II) = RSV/HAH group.

Discussion

After orthodontic treatment, various problems can be encountered, such as white spot lesions (26), oral hygiene problems (27), and relapse, representing one of the most important post-orthodontic problems. While several novel approaches inhibit osteoclastogenesis to lower the risk of post-orthodontic relapse, local distribution of an osteogenic agent through a drug delivery system is probably the most inventive approach (28). The use of hydrogels as extended-release vehicles could be an alternative to solve the main problems of post-orthodontic relapse (9,29,30). Rabbits were

chosen for this experiment because they represent an ideal animal for obtaining a clear picture of bony changes under stress. They have shorter development times and quicker bone turnover than primates (31).

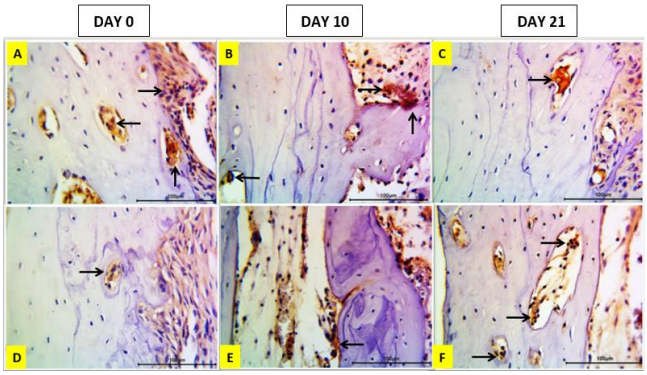

Figure 8: Immunohistochemistry expression of the TRAP 5b in the lower left central incisor at the coronal level of rabbit from the control (upper panel) and RSV/HAH (lower panel) groups on day 0, 10, and 21 post-orthodontic appliance removal reveals weak positive reaction (Score 1+) in the RSV/HAH group on day 0 (D). A moderate positive reaction (Score 2+) was observed in the control group on days 0 and 21 (A&C) and in the RSV/HAH group on days 10 and 21 (E&F). A strong positive reaction (Score 3+) occurred in the control group on day 10 (B). The positive reaction is the dark brown color of the osteoclasts (arrows). 400X.

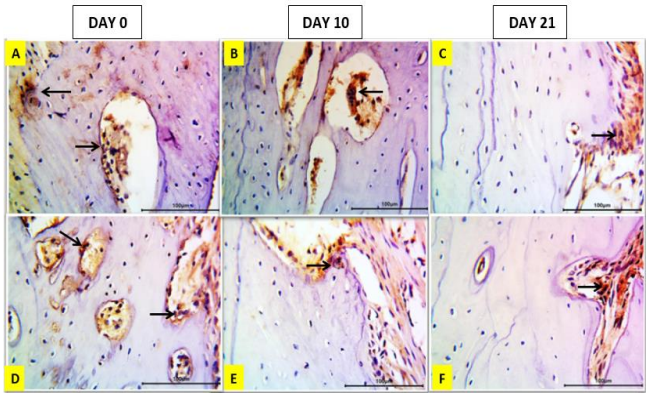

Figure 9: Immunohistochemistry expression of the TRAP 5b in the lower left central incisor at the apical level of rabbit from the control (upper panel) and RSV/HAH (lower panel) groups on day 0, 10, and 21 post-orthodontic appliance removal reveals weak positive reaction (Score 1+) in the RSV/HAH group on day 0 (D). A moderate positive reaction (Score 2+) was observed in the control group on days 0 and 21 (A&C) and in the RSV/HAH group on days 10 and 21 (E&F). A strong positive reaction (Score 3+) occurred in the control group on day 10 (B). The positive reaction is the dark brown color of the osteoclasts (arrows). 400X.

In this study, rosuvastatin, a synthetic, hydrophilic, powerful, and very effective statin, was applied locally by hyaluronan hydrogel to reduce post-orthodontic relapse. Prior research has demonstrated that hydrophilic statins work better than lipophilic ones in mineralization and proliferation (32). The dose of RSV is based on the findings of ÖZER *et al.* (8), who applied 1 mg of RSV locally to improve bone in a rabbit model and found that this increased the volume of new bone and total bone volume.

The histological findings for the RSV/HAH group versus the control group showed a significant increase in osteoblast counts on day 0 and day 10 both coronally and apically, due to the ability of local RSV to encourage osteoblastogenesis (8,33). The histological findings of both groups revealed that the largest osteoblast count was on day 0, followed by a gradual decline on day 10, reaching a minimal count on day 21. On day 0, the working field represents an area of tension during the retention period where osteoblasts are differentiated to increase retention, while on day 10, relapse was started and accompanied by a reduction in the osteoblast count consistent with previous studies (17,18).

A significant reduction in osteoclast counts on day 0 and day 10 was found in the RSV/HAH group both coronally and apically. Reducing osteoclast activity and, consequently, bone resorption may directly reduce orthodontic tooth movement during relapse. This finding agrees with previous studies that used local RSV on bone and found that it creates potent effects inhibiting osteoclastic bone resorption (8,33). The largest osteoclast count was found on day 10 in both groups because the working field during relapse represents the pressure side that is associated with increased osteoclast count and bone resorption (18). The blood vessel count increased significantly on day 10, which supports the role of RSV and different statins in angiogenesis by increasing the release of important angiogenetic factors, such as VEGF (8,28). This can promote bone differentiation and remodeling that aid in post-orthodontic relapse reduction.

A similar effect was observed at both the coronal and apical levels of the working field, which means that the local injection of RSV/HAH was effective and involved the entire area of injection, which aids in the positive effect of the material in post-orthodontic relapse as in agreement with Yaseen *et al.* (15). On day 21, most bone remodeling was complete, and the teeth were almost stable, which may explain the non-significant difference on day 21 in all the evaluated variables. This agrees with previous studies, which found the lowest number of osteoblasts, osteoclasts, and blood vessels in the last observation period (17,34,35). So, the most prominent results were observed on day 0 and day 10.

The immunohistochemical stain of the bone section at the W.F. revealed a positive reaction for BAP and TRAP 5b in both groups, coronally and apically, with different intensities. They represent the biomarkers for osteoblasts and osteoclasts, which are important for bone remodeling during

orthodontic treatment (1). Immunohistochemical findings are consistent with histological findings. BAP is a glycoprotein found on the osteoblast's surface and indicates the biosynthesis activity of these bone-forming cells. It has been confirmed to be a sensitive and dependable bone metabolism measure and a good biomarker for bone formation (36). It is an indicator of osteoblast metabolism and represents a specific marker for osteogenesis (37). The result of the current study showed that the expression of BAP in the RSV/HAH group was strong (score 3+) on day 0, moderate (score 2+) on day 10, and weak (score 1+) on day 21. The significant increase in BAP expression in the RSV/HAH on day 0 and day 10 agrees with previous studies that used (1 mg) RSV on bone and found that it can greatly affect bone apposition, boost bone regeneration, and increase total bone volume by increasing BMP-2 expression and alkaline phosphatase activity (8,33,38). Rezazadeh *et al.* (39) found that RSV/injectable hydrogel could sustain osteoblast proliferation in cell culture and had great potential applications in bone tissue engineering. The expression of BAP is consistent with the histological findings of this study, which found an increase in osteoblast count on day 0, followed by a gradual decrease, reaching the minimal osteoblast count on day 21.

TRAP 5b has been indicated as a specific and sensitive marker enzyme of osteoclasts and represents a reliable marker of osteoclast number. TRACP 5b levels have been proposed as a potential indicator of bone resorption state (40). The current study showed a significant reduction in the expression of TRAP 5b on day 0 from moderate expression (score 2+) in the control group to weak expression (score 1+) in the RSV/HAH group. Also, there was a significant reduction in the expression of TRAP 5b on day 10 from strong expression (score 3+) in the control group to moderate expression (score 2+) in the RSV/HAH group, both coronally and apically. This finding agrees with previous studies that used local RSV on bone and found it creates potent effects inhibiting osteoclastic bone resorption (8,33). TRAP 5b was at its highest level (score 3+) with strong expression on day 10 in the control group, both coronally and apically, because at this time, the working field during relapse became a pressure side that is associated with an increase in the osteoclast count and bone resorption (18).

Several experiments were conducted to investigate the mechanisms of bone anabolism modulated by statins (41-43). It was shown that statins can, through several methods, decrease osteoclastogenesis, suppress osteoblast apoptosis, and enhance osteogenesis. Statins encourage osteogenesis by upregulating the expression of BMP-2 and Runx2. Additionally, statins stimulate the TGFβ/Smad 3 pathway, inhibiting osteoblast apoptosis. Furthermore, statins can modulate the OPG/ RANKL/ RANK system to inhibit osteoclastogenesis (42,43). Also, Monjo *et al.* (44) found that, in addition to the capability of RSV to promote osteoblast differentiation, it controls the expression of the Slco1a1gene, which may create the transport system for RSV across the cell membrane in the mature osteoblasts.

The hyaluronic acid hydrogel was selected in this study based on the previous studies that applied it as a drug carrier (15,45) and due to its ability to enhance mineralization and osteogenesis (45-47). The histological findings of previous study established that H.A. could increase bone formation ability and reduce bone resorption by increasing vascularization and osteoblast count, as well as by reducing the activity of osteoclast, which is important for orthodontic tooth movement (48). Compared to other drug carriers, H.A. showed better results in bone regeneration (49). When Glycosil® and Heprasil®, two different commercially available H.A. hydrogels, were compared, Glycosil®, due to its two-phase release ability, formed more and better bone quality in vivo than Heprasil® (50).

A dual osteoinductive effect of the RSV/HAH formula used in this study was expected because of the combined effects of HAH, which can improve mineralization and osteogenesis, and RSV, which can stimulate bone formation (47). This agrees with Ibrahim and Fahmy (51), who get the advantages of RSV and the drug carrier (chitosan) in wound and bone regeneration. I also agree with Rezazadeh *et al.* (39), who found that loading RSV into a Pluronic F127/hyaluronic acid hydrogel led to advancements in osteoblast proliferation. Akbari *et al.* (52) found that the RSV loaded by thermosensitive hydrogel could be used for bone defects such as osteoporosis and bone fractures. The use of hydrogel as a drug carrier in orthodontics and for bone regeneration is promising. Future investigations are required to find the best osteogenic agent/hydrogel formula and evaluate its effect before application in human trials (29).

Conclusions

Local injection of RSV/HAH is useful for enhancing alveolar bone regeneration. It results in a significant increase in osteoblast and blood vessel count and a reduction in osteoclast count accompanied by elevation of BAP and reduction of TRAP 5b. These histological and immunohistochemical findings that enhance bone regeneration may help reduce relapse after orthodontic tooth movement.

Acknowledgement

The author thanks the College of Dentistry, College of Veterinary Medicine, and College of Education for Pure Science at the University of Mosul for their support in this research.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

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تأثير الروزوفاستاتين/هايلورونان هايدروجيل الموضعي على تقليل الانتكاس التقويمي في الارانب: دراسة نسيجية ومناعية نسيجية كيميائية

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الخلاصة

يمثل الحقن الموضعي للأدوية المرممة للعظم محمولا على حامل دواء أحد أحدث الطرق لتقليل الانتكاس التقويمي. هدف الدراسة هو دراسة تأثير الحقن الموضعي للروزوفاستاتين محمولا على حامل الدواء (الثايوليتيد هايلورونك اسيد هايدروجيل) على اعادة تشكيل العظم. في اليوم الأول من فترة التثبيت بعد حركة تقويم الأسنان للقواطع السفلية للأرانب، تم حقن ١٥ أرنب ألبينو موضعيا بـ (٢٠٠ مل) من المحلول الملحي المخزن بالفوسفات، و ١٥ أرنبًا تم حقنهم بـ (٢٠٠ مل) من الروزفاستاتين محمولا على الهايدروجيل. تقييم التأثير النسيجي للمادة على إعادة تشكيل العظم تم بتقييم عدد خلايا البناء والهدم والأوعية الدموية كذلك تم إجراء الاختبارات النسيجية الكيمياء المناعية لتحديد تعبير ال (الفوسفاتيز القلوي العظمي) وحمض الفوسفاتيز المقاوم للترترات في منطقة الحقن على ثلاث فترات زمنية (٢١،١٠،٠ يوم) وعلى مستويين التاجي والجذري. التحليل الاحصائي تم باستخدام برنامج الاحصاء (عند مستوى دلالة ٠,٠٥). أظهر كروب الروزوفاستاتين/ هايلورونك اسيد هايدروجيل زيادة معنوية في الخلايا البانية للعظم وتعبير الفوسفاتيز القلوي العظمي (تقييم ٣+) في اليوم ٠ و(تقييم ٢+) في اليوم ١٠، نقصان معنوي في أعداد الخلايا الهادمة للعظم، مع تقليل ملحوظ في تعبير حمض الفوسفاتيز المقاوم للترترات (تقييم ١+) في اليوم ٠ و (تقييم ٢+) في اليوم ١٠ مع زيادة ملحوظة في الأوعية الدموية في المستويين التاجي والقمي. كاستنتاج: يظهر الحقن الموضعي لل الروزوفاستاتين/ هايلورونك اسيد هايدروجيل تأثيرات واعدة على إعادة تشكيل العظام السنخية من خلال زيادة تعبير الفوسفاتيز القلوي العظمي، وأعداد الخلايا البانية للعظم والأوعية الدموية وتقليل تعبير حمض الفوسفاتيز المقاوم للترترات وأعداد الخلايا الهادمة للعظم.