



## Local Rosuvastatin Loaded by Thiolated Hyaluronan Hydrogel for Post orthodontic Relapse Reduction. In Vitro Preparation and In Vivo Assessment in Rabbit



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### Abstract

**B**ACKGROUND: Relapse after orthodontic treatment represent an unresolved orthodontic problem. Local injection of osteogenic agents loaded by drug carrier serves as a key strategy for developing materials that may efficiently affect bone remodeling and reduce orthodontic relapse. Aims: The objective of this study was in vitro loading, preparation, and characterization of Rosuvastatin on injectable thiolated hyaluronan hydrogel with assessment of its clinical effect. Material and Method: Rosuvastatin (RSV) was loaded into injectable thiolated hyaluronan hydrogel (HAH) with in vitro assessment of the resultant material by evaluation of <sup>1</sup>H-NMR, FTIR and SEM, injectability, gelation behavior, swelling behavior and release kinetics of RSV. In vivo evaluation was done by local injection of experimental materials in New Zealand male rabbits' model after orthodontic treatment. The rabbits were randomly divided into two groups: group I (control) injected with 200 µl of phosphate-buffered saline (PBS), group II injected with 200 µl of RSV/HAH, the effect on relapse was assessed by measurement of relapse amount and percentage after orthodontic treatment. Statistical analysis was performed by independent sample t-test (P < 0.05 was considered significant). Results: Loading RSV into HAH was confirmed by <sup>1</sup>H-NMR and FTIR. RSV/HAH showed a significant reduction in relapse amount (1.70mm, 1.78mm) and relapse percentage (53.4%, 55.9%) compared to control which was (2.65mm, 2.79mm) and (85.2%, 89.7%) on days 17 and 21 respectively. Conclusions: HAH was successfully prepared for local RSV delivery. RSV/HAH has the potential to enhance post orthodontic teeth stability and could have the ability to reduce relapse after local injection.

**Keywords:** Rosuvastatin, Injectable Thiolated Hyaluronic Acid Hydrogel, Post Orthodontic Relapse, Rabbit Model.

### Introduction

The cause of post orthodontic relapse movement is unknown because it is a complicated and multifactorial process. Alveolar bone remodeling is a crucial component of relapse processes. After orthodontic movement, immature bone needs to be replaced by mature bone which is more stable and more resistant to resorption [1]. Pharmacological medicines can effectively prevent relapse when administered systemically [2] but doing so has side effects that can affect the entire skeleton, including an increase in bone mineral density [3]. Local application could be the best to treat periodontal bone loss. Additionally finding a proper way to

deliver the drug with optimal bioavailability remain a major challenge [4], [5].

Statins, such as simvastatin, atorvastatin, lovastatin, pravastatin and rosuvastatin were mainly developed to decrease the plasma levels of cholesterol and prevent its production [6]. Rosuvastatin (RSV) is a new, third generation, synthetic, hydrophilic, highly efficacious statin. RSV has pleiotropic properties, such as the ability to stimulate bone, enhance vascularization, and reduce inflammation. It is more potent in comparison to simvastatin and atorvastatin [7]. When applied locally, RSV have potent effects that increase alkaline phosphatase activity and Bone morphogenetic protein 2 (BMP-2) expression, which

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DOI: 10.21608/EJVS.2024.283931.2021

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promote osteoblastic development and inhibit osteoclastic bone resorption [8]. According to systemic review based upon the animal studies regarding the effects of Statins on relapse after orthodontic treatment, Afshari *et al.* [6] found that the effect of Statins on relapse after orthodontic treatment remains debatable as some studies registered decrease in orthodontic relapse after statins administration, while others illustrated non-significant changes.

Among all the conventional approaches, and in the presence of several new methods for blocking osteoclastogenesis and stopping orthodontic relapse, the most innovative and well-managed method is probably local drug administration via a drug delivery system since it offers the best control [5], [9].

Hydrogels are water-swollen, three-dimensional chemically crosslinked scaffolds, having exceptional biocompatibility and the capacity to integrate cells, medications, and tiny particles [10]. Hyaluronic acid (HA) is widely used to prepare biomaterials for tissue engineering [11]. It is a naturally occurring glycosaminoglycan (GAG), a polysaccharide of high molecular weight that exhibits fascinating properties. Recently, HA is becoming a more popular biopolymer as a starting material for hydrogel designs because of its biocompatibility, biodegradability and adaptability also due to its unique properties resembling living tissues that make this hydrogel attractive carriers for the localized and targeted delivery of various drugs [12], [13].

Modified Thiolated Hyaluronan Hydrogel (HAH) has been developed as an injectable matrix to heal bone and articular cartilage defects. This system is usually in a solution-state before administration that can undergo gelation under physiological conditions after injection. It can be injected in liquid form avoiding surgical implantation into tissue with minimal invasiveness [14].

According to the best knowledge of the researchers, there is no available data about the effect of local administration of RSV alone or about the effect of local administration of RSV loaded by injectable hyaluronan hydrogel on the post orthodontic relapse prevention. Also, no available data about the use of injectable thiolated hyaluronan hydrogel as drug carrier in orthodontic treatment.

The aim of this study was the preparation and loading of RSV on injectable thiolated hyaluronan hydrogel with *in vitro* evaluation of the injectable materials also to evaluate its clinical effect in post orthodontic relapse prevention in rabbit model. Therefore, we hypothesized that this injectable hydrogel drug combination could stimulate bone

tissue regeneration which may be helpful in post orthodontic relapse reduction.

### **Material and Methods**

#### *Loading Rosuvastatin into Injectable Thiolated Hyaluronan Hydrogel:*

Rosuvastatin (Awamedica Erbil, Iraq) was loaded into thiolated hyaluronan hydrogel (Advanced BioMatrix, USA) seen in Figure(1) for the preparation of injectable material. For loading RSV (1mg)/200µl for each rabbit and according to manufacturer instructions, allow Glycosil® to come at room temperature for 1 hour. 1 mL of deionized degassed water was added to one vial of Glycosil®. Immediately vortex each vial for a few seconds after the addition of water. Once the solid was dissolved, this solution was added into a previously sterilized vial containing RSV suspension. Besides and according to manufacturer instructions, 0.5 mL of deionized degassed water® was added to the thiol reactive crosslinker vial (Extralink-Lite®) and was shaken until complete dissolution. Subsequently, 0.25 mL of crosslinker was poured into the Glycosyl-drug mixture. A 3D hydrogel was formed when Extralink-Lite® was added to Glycosil® in a 1:4 volume ratio (0.25 mL Extralink-Lite® to 1.0 mL Glycosil®), this was mentioned in the direction of use of the Glycosil®. The resultant prepared injectable materials (HAH and RSV/HAH) were used for the *in vitro* investigation and *in vivo* injection.

#### *In Vitro Characterization and Evaluation of The Injectable Hydrogels:*

##### *<sup>1</sup>H-NMR and FTIR :*

Structural evaluation of the hydrogel's formulations (HAH and RSV/HAH) was done using <sup>1</sup>H-NMR (AVANCE NEO400, Bruker, Germany) and FTIR (Bruker Optics, Germany) [15],[16], [17].

*Scanning Electron Microscopy (SEM):* The surface morphology of the injectable hydrogels (HAH and RSV/HAH) was determined using Scanning Electron Microscopy (ZEISS EVO10 SEM, Germany) after being lyophilized using OLABO Freeze Dryer Lyophilizer (OLABO, China) and gold spraying for better conductivity [18].

##### *Evaluation of Injectability:*

The injectability (HAH and RSV/HAH) was evaluated using Insulin syringe with different gauges needles (30,27,25,23 and 22 gauge) [18], [19].

##### *Evaluation of Gelation Behavior:*

The gelation behavior of (HAH and RSV/HAH) was evaluated by measuring gelation time at different temperatures (at room temp= 22 °C and at 37 °C).

Vial tilting method was used for the evaluation of gelation time [20]. Gelation time was considered at the point when there was no flow of the hydrogel for more than 1 min after repeatedly inverting each vial that contained HAH and RSV/HAH solutions.

#### *Swelling behavior:*

Swelling ratio of (HAH and RSV/HAH) was measured by immersing each hydrogel formula in PBS under 37 °C [19]. At the end of each incubation period in PBS (0.5, 1, 6, 24, 48, 72 h and 7 days), the hydrogel was dried using filter paper. The results were recorded until equilibrium was maintained [21]. The following formula was used to calculate the swelling ratio (SR):

$$\% \text{ SR} = (\text{W}_{\text{wet}} - \text{W}_{\text{dry}}) / \text{W}_{\text{dry}}$$

where  $\text{W}_{\text{wet}}$  is the hydrogel's final mass following swelling in PBS and  $\text{W}_{\text{dry}}$  is the hydrogel sample's initial mass [20], [22].

#### *In Vitro Release Kinetics of RSV:*

After loading RSV into HAH and gel formation, in vitro release of RSV from HAH was performed using dialysis method. Presoaked dialysis membrane (Special Products Laboratory, USA) was filled with 1 mL of RSV/HAH. The dialysis bag was then placed in 150 mL release medium (PBS) at pH (7.4) and temperature (37°C) [23].

Release test samples (1ml) were taken and analyzed over (15, 30, 60, 90 & 120 min) in the first day after drug loading. Then, (1 ml) release test samples were taken and analyzed over (1, 2, 3, 7, 14 and 21 days) after 5 min agitation at 100 rpm for the PBS using hot plate stirrer, while the same amount of PBS solution was added to the release system [24]. The temperature for PBS was kept steady over the study period. The amounts of RSV released into the PBS beaker was measured using Spectrophotometer (UV-1800, Shimadzu Corporation, Japan) at  $\lambda_{\text{max}} = 230.5\text{nm}$  and  $250.5\text{nm}$ .

#### *In Vivo Evaluation of The Injectable Hydrogels on Post orthodontic Relapse:*

##### *Animal samples and experimental groups:*

All guidelines and experimental protocols for this study were approved by the "Institutional Animal Care and Use Committee, College of Veterinary Medicine, University of Mosul, Ministry of Higher Education and Scientific Research, Iraq." With the approved REC reference number, UM. VET. 2023. 094.

For in vivo evaluation of the ability of RSV/HAH for post orthodontic relapse prevention, a total sample of 10 adult male albino rabbits aged 5–8 months with average weight of approximately 1000 g ranged from 950 g to 1150 g. The formula that utilized to establish sample size was as following :  $[n = (z \ r/D)^2]$ . (95% confidence) , n = required sample size, z (constant)= 1.96 units, r = (precision) = 0.2 units [2], [25]. The final sample size was

calculated after adjustment and the final sample size = (z)\ group. According to this, the estimated sample size for each group was five animals. Rabbits were randomly divided into 2 Groups as following:

Group I (control negative): rabbits received orthodontic appliance and were injected with 200  $\mu\text{l}$  solution of phosphate-buffered saline (PBS).

Group II (RSV/HAH): rabbits received orthodontic appliance and were injected with 200  $\mu\text{l}$  injectable thiolated hyaluronan hydrogel drug carrier loading by (1mg) RSV.

#### *Insertion of Orthodontic Appliance:*

Modified orthodontic appliance using bands with bondable tube (Dentaurum, Germany) mounted into rabbit incisor teeth [26]. Nickel-Titanium open coil spring (IOS, USA) of 6 circles and about 6.5 mm in length was inserted along 0.016"×0.022 stainless-steel wire (IOS, USA) between the two tubes. The two bands ligated with each other with stainless steel ligature wire (Dentaurum, Germany) to compress the open coil from 6.5 mm to 3.3 exerting a lateral reciprocal force of about 50g [27] measured by stress and tension gauge (China).

General anesthesia was used before appliance insertion by giving the rabbits intramuscular injections of a combination of (50 mg/kg body weight) ketamine hydrochloride (SIR ALDAWA CO, Baghdad, Iraq) and (10 mg/kg body weight) xylazine hydrochloride (Interchemie- Holand) [28]. Orthodontic appliances fixation were done using Ortho Bite Light Curing Composite (FGM, Brazil). Finally, orthodontic force was used to move the lower incisors of rabbits distally by freeing the bands from the ligature wire, allowing the bands to become active and start tooth movement for two weeks [28]. After the two weeks, the springs were opened and inactive and a three weeks retention period was started.

#### *Drug Administration and Injection Site:*

On the first day of the retention period, the injection was administered with the specified drug for each group after general anesthesia. Submucosal injection was delivered using disposable insulin syringe with a 23-gauge needle. The total amount injected was 200  $\mu\text{l}$ . It was inserted into the oral mucosa, in the muco-gingival junction, through the attached gingiva, close and parallel to the mesial surface of the experimental side (left mandibular incisor). 100  $\mu\text{l}$  was injected into the labial aspect and 100  $\mu\text{l}$  into the lingual aspect of the vestibular mucosa shown in Figure(2). All injections were volumetrically equivalent and were administered one time [27], [28].

#### *Tooth Movement Measurement:*

To determine the amount of relapse, interproximal space between the lower central

incisors at the level of mesial tip was recorded using an electrical digital (China) as shown in Figure (3).

The mean of gained space (GS) at the time of orthodontic appliance removal and the remained space (RS) on days (0, 3, 17 and 21) after the retention period was recorded. The relapse distance (RD) was equivalent to (GS-RS) for all experimental groups. The percentage (%) of relapse distance [(GS-RS)/GS X 100] on days (0, 3, 7, 17 and 21) after the retention period was also determined [27], [28].

#### *Statistical analysis*

Shapiro-Wilk test was employed to verify the normality of the data distributions. Statistical analysis of the data for the relapse distance and relapse percentage between the two groups was performed by independent sample t-test.  $P < 0.05$  was considered significant. Statistical analysis was performed using IBM SPSS Statistics, Version 26 (IBM Corporation, USA).

### **Results**

#### *Results of In Vitro Preparation and Characterization:*

**<sup>1</sup>H-NMR & FTIR Results:** A homogenous injectable hydrogel can be obtained before drug loading and after loading RSV suspension into the HAH.

From the <sup>1</sup>HNMR examination of HAH seen in Figure (4), that there is a strong signal at 3.799 sigma due to the protons of the water being very strong, as their intensity hides the hydrogel bands. This will be useful when mixing RSV with HAH, as it will work to give a final diagnosis of the mixing process, was it done through a chemical reaction or just mixing.

The <sup>1</sup>HNMR examination of RSV/HAH seen in Fig.(5), showed the presence of water bouton bands in the same place and intensity at 3.738  $\delta$ , and the rest of the RSV bands appeared at multiple bands at 2.9  $\delta$  up to 3.506  $\delta$ , due to the CH<sub>3</sub> protons present on with draw groups in the compound, and also the appearance of the CH<sub>3</sub> band next to the sulfone 4.516  $\delta$ , as well as the ben-zene ring bands, which It gave a split binary signal dd at frequencies of 6.317-6.709  $\delta$  and 7.293-7.717  $\delta$ , and the acid packet disappeared because the acid was in the form of a salt (drug) due to its ease of dissolving in water, which supports that mixing and loading of the drug substance on the hydrogel occurred and that any chemical reaction did not occur.

From FTIR spectrum for HAH seen in Figure (6), we can notice the appearance of the aliphatic CH stretch band at 2889 cm<sup>-1</sup>, as well as the amide C=O band at 1614 cm<sup>-1</sup>, and that the large amount of solvent has overwhelmed the NH band, which appears within the broad band of solvent (water) in The region is from 3100 to 3500 and is marked by two main peaks, which are 3269 and 3372 cm<sup>-1</sup>, in

addition to the rest of the basic aliphatic bands for bending CH, as well as C-N and the rest of the bands.

From the FTIR spectrum of RSV/HAH seen in Fig.(7), we can notice that the basic bands of the hydrogel match, where the aliphatic CH stretch band appears at 2889 cm<sup>-1</sup>, as well as the amide C=O band at 1650 cm<sup>-1</sup>, and the acid C=O band appears with the same original value for the compound at 1599. cm<sup>-1</sup>, and with a lesser intensity. Also, the appearance of the C=N ring at 1523 cm<sup>-1</sup>, and the S=O stretching bands at the frequency of 1509 cm<sup>-1</sup>, in addition to the aliphatic CH bending bands and the C-O stretching band for the distinct secondary alcohols at the stretching frequency of 1150 cm<sup>-1</sup>, and the appearance of a characteristic stretching band C-F is at the position of 961 cm<sup>-1</sup>.

**SEM Results:** Structural & morphological observations for HAH and RSV/HAH were performed using SEM at 10, 20 and 100  $\mu$ m magnification shown in Figures (8 and 9) respectively.

**Injectability Results:** HAH before and after RSV loading could be easily injected and squeezed out of a syringe with a 23 gauge needle.

**Gelation Behavior Results:** Vial tilting test shown in Figure (10) showed that gelation time of HAH & RSV/HAH decreased by increasing temperature from room temperature (22°C) to body temperature (37°C). The gelation time was reduced from (40min-10min) and (180-22min) respectively by increasing temperature from 22 °C to 37°C. While gelation time of the hydrogel was increased after being loaded with RSV drugs from 40 min for blank hydrogel (HAH) to 180 min for RSV/HAH at 22°C and increased from 10 min for HAH to 22 min for RSV/HAH at 37°C.

**Swelling Behavior Results:** The swelling ratio was 5.95% and 5.84% for HAH and RSV/HAH and respectively within 48hr.

**Release results:** The release profile of RSV from HAH showed a two-phase release. RSV showed an initial burst of 69.2% during the first 15 min and 76.7% during the first 24 h, followed by a steady release over 1–21 days. Up to 95.2% of the RSV was released over 21 days.

#### *In Vivo Effect of The Injectable Hydrogels on Post orthodontic Relapse:*

Relapse distance (RD) was calculated by the equation (GS-RS) when gaining space (GS) was about (3mm) at the time of appliance removal and (RS) represent the remaining space after the retention period. Statistical analysis between the two experimental groups showed that there is a significant difference in (RD) and relapse% between the two groups on the last two measurements on (17& 21) days showed in (Table 1). RSV/HAH showed significant reduce in the RD and relapse%

which was (53.4&55.9)% on days (17&21) respectively compared with control group that showed (85.2&89.9) % of relapse on days (17&21).

### **Discussion**

#### *In Vitro Preparation of the Injectable Hydrogels:*

<sup>1</sup>H NMR&FTIR: A homogenous injectable hydrogel can be obtained before drug loading and after loading (1 mg) RSV suspension which is important physical properties of the hydrogels.

<sup>1</sup>H NMR spectrum is very important to give a final decision about mixing RSV with HAH, as the signals for identifying the mixture give a final diagnosis of the product and whether the process was loading or resulted in a chemical reaction [29]. <sup>1</sup>H NMR confirmed the loading process, the chart showed that the drug substance was mixed and loaded onto the hydrogel and that a chemical reaction did not occur, as the bands of the mixture matched what was obtained for the materials before mixing, and no new signals were produced from the mixing process [30]. The interpretations of the above spectra are consistent with previous studies [31-33].

From FTIR beams in comparison between two chart for HAH and RSV/HAH, it was confirmed that the process of mixing RSV with HAH is considered as loading and that no chemical reaction or interference occurred as a result of the loading process [34],[31].

These data confirmed that RSV was successfully loaded into the HAH without any change in the chemical structure of both as the bands of the mixture matched what was obtained for the materials before mixing, and no new signals were produced from the mixing process.

In this work, thioleted hyaluronic acid hydrogel, or Glycosil® was utilized. HA is a biocompatible and chemically adaptable polymer that may be used to create clinical-grade hydrogels that imitate the extracellular matrix and have a variety of biological and mechanical characteristics for the delivery of molecules and cells [21]. The addition of thiolated derivatives (PEGDA)® is beneficial for crosslinking. Drug delivery systems that are more stable and effective are produced when thiol groups are protected by disulfide bonds or crosslinking, which enhances the characteristics of thiomers [35].

*SEM:* Structural or morphological characterization is one of the most popular methods for characterizing injectable hydrogels, which allows for the observation of the hydrogel's porous microstructure. The capacity of the hydrogels to swell is closely correlated with this microstructure because of the water entrapment that occurs there. In this study, morphological observations were performed on freeze-dried hydrogel samples using SEM.

*Injectability:* HAH before and after RSV loading could be easily injected and squeezed out of a syringe with a 23 gauge needle. Since a 23 gauge needle is frequently employed in minimally invasive methods for both human clinical trials and preclinical animal investigations, it was selected for numerous experiments [36]. The viscosity of HAH solution made it easy to be injected through the 23 gauge needle while not allowed to it to be injected through smaller needle.

*Gelation Behavior:* In the early stage after mixing the hydrogel components, the system showed a high degree of liquidity because of the cross-linking reaction not having started yet, supplying the hydrogel with a desired injectability for use. A hydrogel cross-linking network began to generate, resulting in the gel's creation. The gelation time of HAH & RSV/HAH was reduced from (40min-10min) and (180-22min) respectively by increasing temperature from 22 °C to 37°C. This agrees with Ghanem, et al. [20] who stated that gelation time was determined by the temperature to which the hydrogel was exposed, and it was reduced by elevating the temperature from room temperature to body temperature. This may be explained as the crosslinking speed is faster as the temperature increase.

In contrast, gelation time of the hydrogel was increased after being loaded with RSV drug from 40 min for blank hydrogel (HAH) to 180 min for RSV/HAH at 22°C and increased from 10min for HAH to 22 min for RSV/HAH at 37°C. This is in agreement with Ijaz, et al. [17] who found that there was an increase in the gelation time after drug loading into the hydrogel. This may be attributed to the change in the concentration of the hydrogel by the addition of drugs that prolong the time required for complete crosslinking reaction.

*Swelling Behavior:* All hydrogel samples reached an equilibrium swelling state within 48hr by reaching a constant water content. The swelling ratio before and after drug loading was close to each other which was 5.95% and 5.84% for HAH and RSV/HAH. This indicated that loading drugs had no effect on the properties of blank hydrogel. Because hyaluronic acid hydrogels are hydrophilic, they can preserve their three-dimensional spatial network morphology even after swelling to an equilibrium volume in water. Hydrogel samples showed swelling owing to the various hydrophilic groups present in the chemical structure of HA, which lead to water absorption and hydration capabilities [37]. Low swelling ratio of the hydrogel samples investigated in this study meets the physiological requirements for tissue repair and reduce the frictional irritation to the surrounding tissue [38], [39].

*Release:* One interesting approach to obtaining the local release of low bioavailable active agents in bone is the incorporation of antiosteoporotic drugs

into porous hydrogel [11]. Drug could be loaded by thiolated hyaluronic acid hydrogels that would be cross-linked to networks and make porous structures. These pores may be used to load drugs, and the hydrogel may enlarge following injection if tissue fluid is present. Such swelling would cause its pores to widen, allowing the medication to diffuse and release gradually [40].

In addition to its therapeutic role, HA serves as a drug release matrix. Recently, regulated release of various medications from HA and its derivatives has been shown to have a number of advantages, including sustained release rates *in vivo*, minimal or negligible toxicity, increased therapeutic effects, and optimal drug concentration management [13].

The release profile of RSV from HAH showed a two-phase release. RSV showed an initial burst of 69.2% during the first 15 min and 76.7% during the first 24 h, followed by a steady release over 1-21 days. Up to 95.2% of the RSV was released over 21 days. A two-phase release kinetics of different pharmacological agents from different hydrogels was found in many researches [41].

The results of this study is in agreement with Bhakta, *et al.* [42] who found that “Glycosil® demonstrated a burst followed by a sustained release phase for BMP-2 with greater bone formation”. The improvement in the bone-forming ability of Glycosil® hydrogels may be largely attributable to the higher burst release followed by sustained release of active agent. In contrast, the lower initial release of from Heprasil® might have delayed the recruitment of osteoprogenitor cells.

Huang & Brazel [43] stated that the occurrence of the burst effect may be related to the hydrogel's highly porous macrostructure (which is determined by the degree of crosslinking), drug diffusion through the pores in proportion to the hydrogel swelling at the start of release, and osmotic pressure in the diffusion membrane. So, an initial burst release of RSV could be attributed to the swelling ability of the hydrogel in contact with water that leads to enlargement of the pore size and drug release.

#### *In Vivo Evaluation of The Injectable Hydrogels:*

While there are several new methods for blocking osteoclastogenesis to reduce post orthodontic relapse, the most innovative and well-managed method is probably local administration of osteogenic agent via a drug delivery system since it offers the best control. The use of hydrogels as extended-release vehicles could be an alternative to solve the main problems of post-treatment relapse [9].

In this study, RSV, which is a synthetic, hydrophilic, potent, and highly efficacious statin carried by HA hydrogel was applied locally for post orthodontic relapse prevention. Previous studies' have shown that hydrophilic statins are more

effective than lipophilic ones in terms of mineralization and proliferation [44, 45]. RSV, when administered locally produces strong effects that inhibit osteoclastic bone resorption and help in osteoblastic differentiation by enhanced BMP-2 expression and alkaline phosphatase activity [8], [46].

The dose of RSV is according to ÖZER, *et al.* [8] who used (1 mg) RSV for local effect on bone in rabbit model and found that RSV when applied locally increases new bone and total bone volume.

In this study, hyaluronic acid hydrogel was selected because previous researches supported its application as a cell and drug carrier [52], besides its potential to augment mineralization and osteogenesis [53]. When comparing HA with other types of carriers, HA showed better results in bone regeneration. Glycosil® hydrogels could induce bone formation even by delivering the lowest doses of active agent as BMP-2, in comparison collagen sponges failed to induce bone formation at this low dose [54].

Bhakta *et al.* [42] compared two different commercially available hyaluronic acid hydrogel (Glycosil® and Heprasil® hydrogels) and found that Glycosil® hydrogel form more and better bone quality *in vivo* than Heprasil® hydrogel and contributed this finding to the fact that Glycosil® releases more BMP-2 initially than Heprasil® with initial burst release followed by sustained release of drug (as showed in this study).

The results of this study agree with previous animal experimental studies that evaluated the effect of local application of (1mg) RSV on bone and found that it can enhanced bone regeneration, increases new bone and total bone volume and strongly affects bone apposition [8], [47]. Rezazadeh *et al.* [48] found that RSV carried by injectable hydrogel could preserve osteoblast viability and proliferation in cell culture and had a great potential application in bone tissue engineering.

The mechanisms of bone anabolism regulated by statins were explored in several experiments. They found that statins can promote osteogenesis, suppress osteoblast apoptosis, and inhibit osteoclastogenesis by different mechanisms. By suppressing FPP synthesis, lowering cellular cholesterol, and triggering the Ras-PI3K/Akt/MAPK signaling pathway, statins promote osteogenesis by upregulating the expression of BMP-2 and Runx2. By reducing GR production, the suppression of FPP synthesis renders Rho and MKP-1 inactive and can eliminate their detrimental effects on osteogenesis. Statins promote the TGFβ/Smad 3 pathway, which suppresses osteoblast apoptosis. The OPG/RANKL/RANK system, on the other hand, blocks osteoclastogenesis [49], [50]. Also, Monjo, *et al.* [51] found that in addition to the ability of RSV

to encourage osteoblast differentiation, it regulates the expression of *Scol1a1* gene, which may constitute the transport system for RSV across the cell membrane in mature osteoblasts.

In this study, local administration of RSV/HAH resulted in a significant decrease in the amount and percentage of post orthodontic relapse in rabbit model in the last two days of measurement (day17&21) while early measurement (day 3) has non-significant effect as the measurements was recorded early after injection and the effect of RSV/HAH was not occurred yet.

In this study, RSV/HAH formula was predicted to have a dual osteoinductive effect because of the combined effects of HAH, which can improve osteogenesis and mineralization with RSV, which can stimulate bone formation [53]. This agrees with Ibrahim and Fahmy [55] who get the benefits of the dual effect of RSV and drug carrier (chitosan) in wound and bone healing and regeneration. Also agree with Rezazadeh et al. [48] who found that the incorporation of RSV into Pluronic F127/hyaluronic acid (PF127/HA) hydrogel led to improvement in osteoblast proliferation and viability. Akbari et al. [56] also evaluated the impact of two different thermosensitive hydrogels loaded with RSV on proliferation and differentiation of human osteoblast-like MG-63 cells. They found that this "RSV/hydrogel formula might be efficiently applied for bone defects such as osteoporosis and bone fractures in the future".

The clinical results in relapse in this study agree with many previous studies that used Statins for post orthodontic relapse reduction [6], [57], [58] particularly that used local Statins for post orthodontic retention [59], [60], [61]. This may be attributed to the previously mentioned positive impacts of Statins on bone remodeling particularly local Rosuvastatin that can increase alkaline phosphatase activity and Bone morphogenetic protein which promote osteoblastic development and

inhibit osteoclastic bone resorption affecting bone remodeling and aids in teeth retention [8], [47], [48]. RSV also can enhance vascularization, and reduce inflammation [7]. In the other hand the use of hydrogels as drug carrier in orthodontics is promising and further studies is required specifically for orthodontic purposes to find the best formula that combine hydrogel with osteogenic agents an evaluated its effect before application in human trials [9].

### **Conclusions**

RSV can be successfully loaded into HAH to be used in post orthodontic relapse reduction. Local injection of RSV /HAH might be a useful method for enhancement of post orthodontic teeth stability as it showed significant reduction in relapse distance and relapse percentage after orthodontic tooth movement.

### *Acknowledgement:*

Many thanks to 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.

*Funding statement:* Self -funding

*Conflict of interest:* None.

### *Ethical Approvale:*

All guidelines and experimental protocols for this study were approved by the "Institutional Animal Care and Use Committee, College of Veterinary Medicine, University of Mosul, Ministry of Higher Education and Scientific Research, Iraq." With the approved REC reference number, UM. VET. 2023. 094.

### *Author`s contribution:*

The first researcher carried out the practical aspect, completed the task of statistical analysis, making tables, and writing. The second researcher participated in designing the research. The third researcher did the chemical analyses and was the corresponding author. All authors have read and agreed to the published version of the manuscript.



**Fig. 1. Thiolated Hyaluronan Hydrogel Kit (Advanced BioMatrix, USA).**



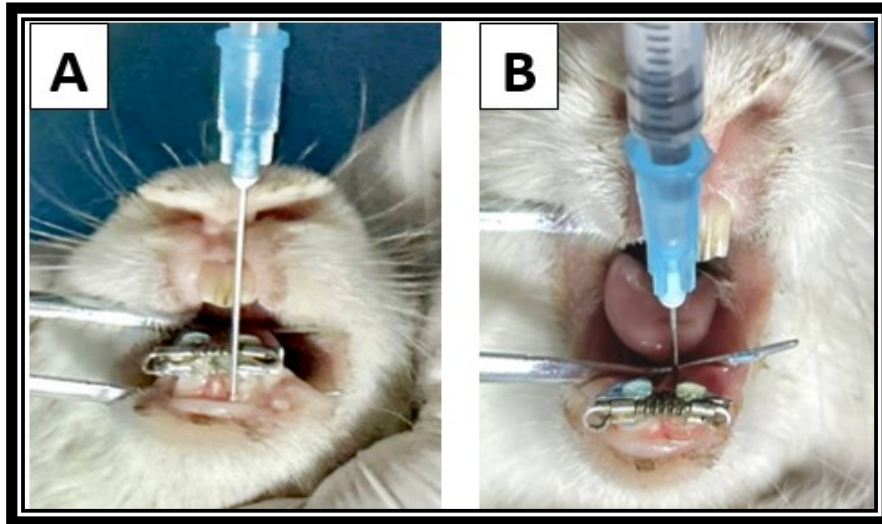


Fig. 2. Local injection of drug A: Labial injection B: lingual injection.

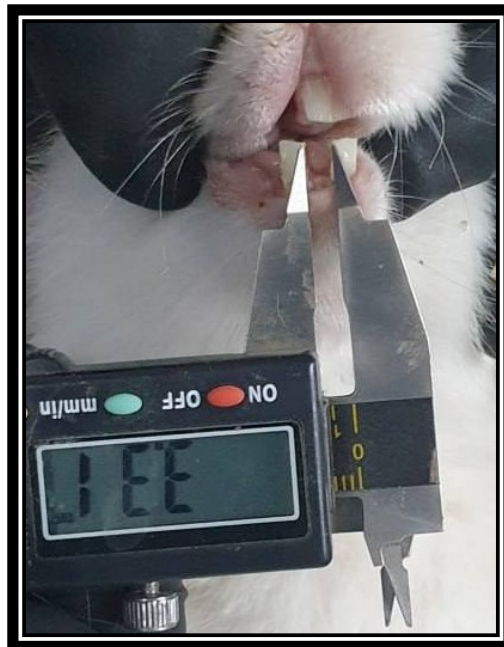


Fig. 3. Direct intra oral measurement of interproximal space with a digital vernier

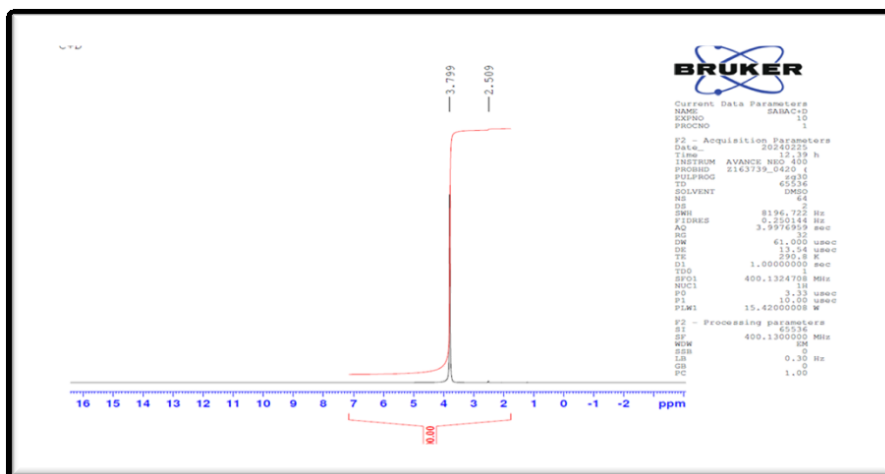


Fig. 4. <sup>1</sup>HNMR of HAH



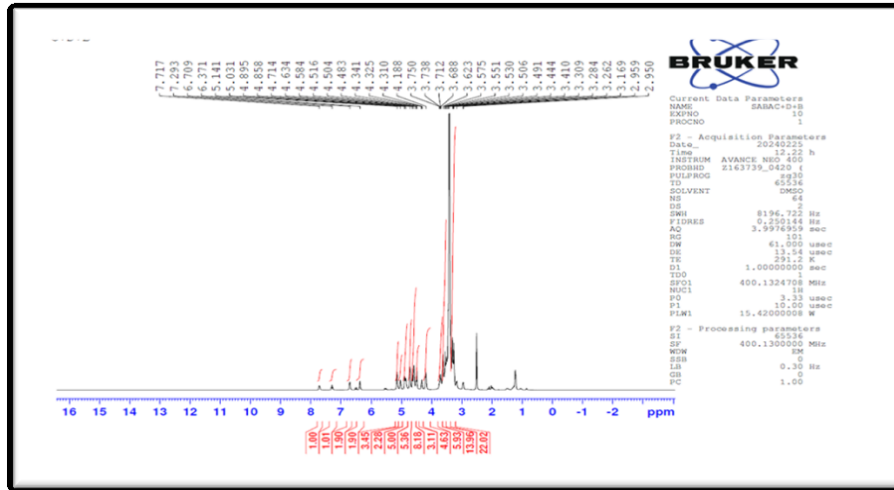


Fig. 5. <sup>1</sup>H NMR examination of RSV/HAH

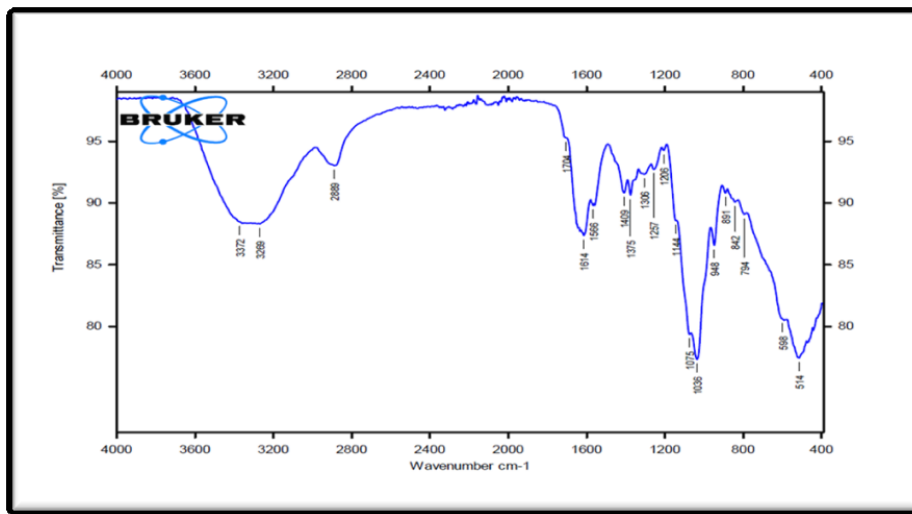


Fig. 6. FTIR of HAH

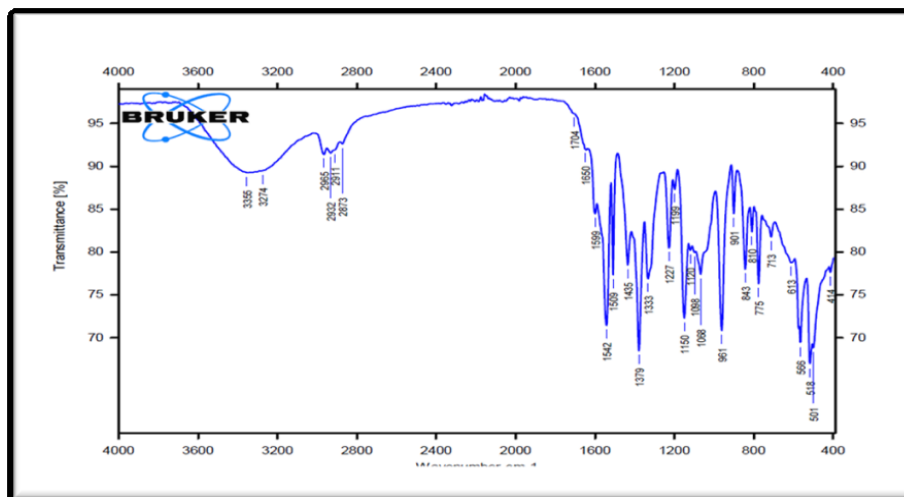


Fig. 7. FTIR of RSV/HAH

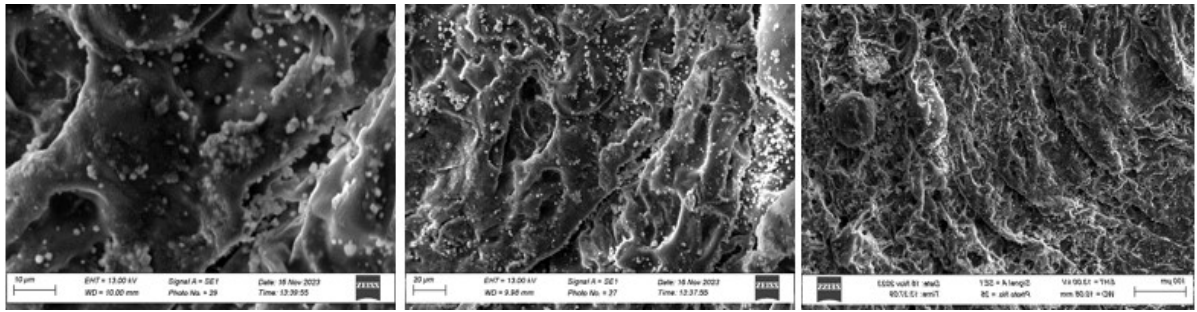


Fig. 8 . SEM Image of HAH at 10, 20 and 100 μm magnification respectively

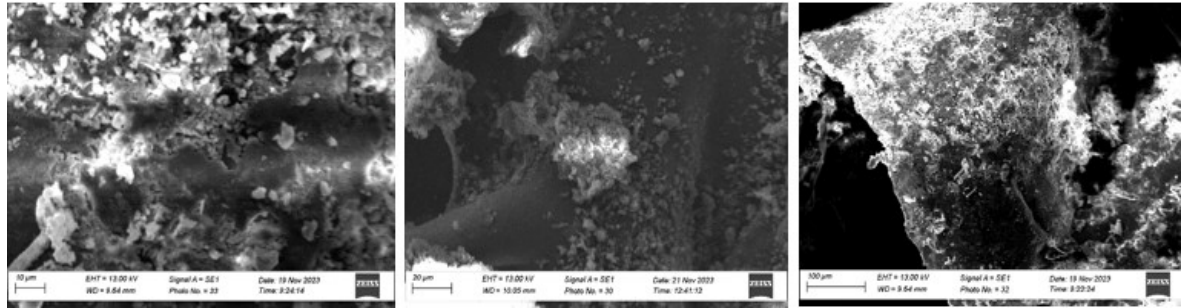


Fig. 9. SEM Image of RSV/HAH at 10, 20 and 100 μm magnification respectively

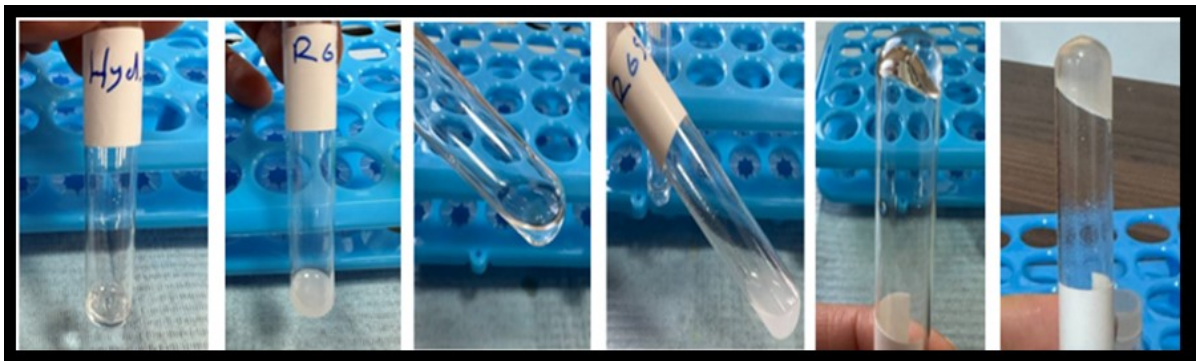


Fig. 10. Vial Tilting Method

TABLE 1. Comparison of the mean of relapse distance between the two groups

Group	Measurement Day	Relapse%	Relapse Mean●	SD*	t-value	P-value**
I	0	-	0.00	-	-	-
II	0	-	0.00	-	-	-
I	3	35.6	1.11	0.094	0.949	0.370
II	3	33.3	1.06	0.098		
I	17	85.2	2.65	0.075	11.044	0.000
II	17	53.4	1.70	0.176		
I	21	89.7	2.79	0.142	10.616	0.000
II	21	55.9	1.78	0.156		

●Mean expressed in mm \*Standard deviation \*\*A significant difference existed at P<0.05

I=Control, II=RSV/HAH

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## الروزوفاستاتين الموضعي محمولا على ( الثايوليتيد هاييلورونك اسيد هايديروجيل ) لتقليل

### انتكاسة الاسنان بعد التقويم. تحضير مختبري وتقييم حيوي في الارنب

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#### المستخلص

**الخلفية:** تمثل انتكاسة الاسنان بعد التقويم مشكلة لم تحل ليومنا هذا يمثل الحقن الموضعي للادوية المرممة للعظم باستخدام حامل دواء مفتاحا لتطوير مواد ممكن ان تقلل الانتكاسة. **الهدف:** تهدف الدراسة الى تحميل، تحضير وتوصيف الروزوفاستاتين على حامل دواء قابل للحقن واجراء اختبارات لفحص المادة في المختبر مع تقييم التأثير السريري للمادة في منع انتكاسة الاسنان بعد الحقن الموضعي في اسنان الارانب. **المواد وطريقة العمل:** تحميل دواء الروزوفاستاتين على حامل الدواء (الثايوليتيد هاييلورونك اسيد هايديروجيل) مع دراسة مختبرية للمادة الناتجة واجراء اختبارات <sup>1</sup>H-NMR, FTIR, SEM ايضا تقييم مواصفات المادة الناتجة واختبار قابلية الحقن والانفخ وتحضير الدواء. ثم التقييم السريري بالحقن الموضعي للمادة في اسنان الارانب بعد التقويم. تقسم الارانب الى قسمين مجموعة ضابطة تحقن موضعيا ب (200 µl) فوسفات بفر سلاين و مجموعة الاختبار تحقن ب (200 µl) من الروزوفاستاتين محمولا على الهيدروجيل. يقدر التأثير السريري للمادة المحقونة على الانتكاسة بقياس كمية الانتكاسة ونسبة الانتكاسة بعد التقويم. التحليل الاحصائي للبيانات بواسطة برنامج الاحصاء SPSS. **النتائج:** نجاح تحميل الدواء على الحامل التي اكدتها اختبارات <sup>1</sup>H-NMR, FTIR. ادى الحقن الموضعي للروزوفاستاتين محمولا على حامل الدواء الى فرق معنوي في كمية الانتكاسة (1.70&1.78mm) ونسبة الانتكاسة (55.9&53.4%) التي كانت اقل بالمقارنة مع المجموعة الضابطة التي كانت (2.65&2.79mm) بنسبة (85.2& 89.7%) في الايام 17 و 21 على التوالي. **الاستنتاجات:** نجاح تحميل الروزوفاستاتين على الحامل (الثايوليتيد هاييلورونك اسيد هايديروجيل) وامكانية استخدامه لتحفيز استقرار الاسنان بعد التقويم حيث له القابلية لتقليل الانتكاسة بعد التقويم بالحقن الموضعي.

**الكلمات المفتاحية:** الروزوفاستاتين ، الثايوليتيد هاييلورونك اسيد هايديروجيل ، انتكاسة الاسنان بعد التقويم ، تقويم الاسنان، نموذج الارنب.