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Vascular Endothelial Growth Factor Expression in Pulp Regeneration Treated By Hyaluronic Acid Gel in Rabbits

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Abstract

The use of scaffolds has created possibilities for the regeneration of tooth structure. Hydrogels made of hyaluronic acid have drawn much attention in regenerative medicine. Consequently, the objectives of the study were to estimate the impact of hyaluronic acid on pulp regeneration using histological evaluation and immunohistochemical localization of vascular endothelial growth factor (VEGF). The entire sample consisted of 20 teeth from ten rabbits, they were used for pulp exposure and coronal pulp tissue removal. After pulp exposure, hyaluronic acid hydrogels were injected into the pulp of the right upper central incisors in the experimental group to promote pulp regeneration. The pulp chambers of the left upper central incisors considered as the control group, and they were filled with temporary filling without the injection of hyaluronic acid. The histological and immunohistochemical evaluations were done in two time periods: the first and second week after pulp exposure. The histological results showed a high mean value (56.3) regarding predentin thickness in the experimental group, especially in the second-week duration, and a significant difference as compared with the control group. The immunohistochemical results disclosed that the experimental group showed strong positive expression for vascular endothelial growth factor in pulpal cells with the highest mean value (34.3) in the second- week duration, with a significant difference as compared with the control group. Hyaluronic acid hydrogel had a supporting function in pulp regeneration, according to the findings of the current study. This was demonstrated by an increase in the expression of VEGF throughout pulpal cells.

Keywords: Pulp regeneration, Hyaluronic acid, VEGF, Angiogenesis, Reparative dentin.

تعبير عامل النمو البطاني الوعائي في تجديد اللب المعالج بهلام حمض الهيالورونيك في الأرانب

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الخلاصة

استخدام السقالات خلق إمكانيات لتجديد بنية الأسنان. جذبت الهلاميات المائية المصنوعة من حمض الهيالورونيك الكثير من الاهتمام في الطب التجديدي. وبالتالي ، كانت أهداف الدراسة هي تقدير تأثير حمض الهيالورونيك على تجديد اللب باستخدام التقييم النسيجي والتوطين الكيميائي المناعي لعامل النمو البطاني الوعائي .(VEGF)تكونت العينة بأكملها من ٢٠ سنًا من عشرة أرانب, تم استخدامها لتعرض اللب وإزالة أنسجة اللب التاجي. بعد تعرض اللب ، تم حقن الهلاميات المائية لحمض الهيالورونيك في غرف اللب في القواطع المركزية العلوية اليمنى في المجموعة التجريبية لتعزيز تجديد اللب. تعتبر غرف اللب في القواطع المركزية العلوية اليسرى مجموعة التحكم ، وتم ملؤها بحشوة مؤقتة دون حقن حمض الهيالورونيك. تم إجراء التقييمات النسيجية والهيستوكيميائية المناعية على فترتين زمنيتين: الأسبوع الأول والثاني بعد تعرض اللب. أظهرت النتائج النسيجية بمتوسط مرتفع (٥٦,٣) في المجموعة التجريبية فيما يتعلق بسمك predentin ، خاصة في الأسبوع الثاني ، مع وجود فرق معنوي مقارنة بالمجموعة الضابطة. أظهرت النتائج الهيستوكيميائية المناعية أن المجموعة التجريبية أظهرت تعبيرًا إيجابيًا قويًا لعامل النمو البطاني الوعائي في خلايا لب مختلفة مع أعلى قيمة متوسطة (٣٤,٣) في الأسبوع الثاني ، مع وجود فرق معنوي مقارنة مع المجموعة الضابطة. كان لهيدروجيل حمض الهيالورونيك وظيفة داعمة في تجديد اللب ، وفقًا لنتائج الدراسة الحالية. تم إثبات ذلك من خلال زيادة التعبير عن VEGFفي جميع أنحاء الخلايا في اللب.

الكلمات المفتاحية: تجديد اللب ، حمض الهيالورونيك ، VEGF، تكوين الأوعية ، عاج ترميمي

Introduction

Tooth caries, trauma, or iatrogenic damage can occasionally reveal dental pulp in clinical settings. Untreated pulp exposure can deteriorate the integrity of the tooth structure and result in pulp necrosis in cases of bacterial infections [(1)]. When dental pulp loses vitality, the tooth becomes more brittle because it can no longer offer nourishment or identify possible infections.

Therefore, teeth that have had RCT treatment are likely to become devitalized, brittle, and vulnerable to postoperative breakage. To treat dental pulp problems, a successful treatment plan is thus required to restore vital tooth pulp. Recent advances related to tissue engineering ensure increasing the opportunities for regenerative endodontic treatment [(2)]. To reestablish or replace the natural activity of organs, tissues , and cells which have been affected by intrinsic or extrinsic sources, a new technique was introduced known as tissue engineering [(3)].

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Previous dental investigation employs tissue engineering techniques and investigates the possibility products as
replacement treatments for tissues such as bone, dentin, and pulp. Regenerative endodontic techniques try to restore biological function by using biological processes to replace lost/damaged dentin-pulp complex tissue [4].

For dental pulp engineering, a number of synthetic scaffolds have been developed, mainly polymers [5] and hydrogels [6]. Earlier advances in cell treatment have identified hyaluronic acid (HA)gel as an important biomaterial in tissue engineering[7,8]. The connective tissue comprises a high level of HA in their extracellular matrix, which makes it a particularly promising scaffold for tissue regeneration[9]. Furthermore, HA supports a variety of biological processes, such as angiogenesis, wound healing, inflammation, and bone repair. Also , HA promotes mesenchymal cell chemotaxis, proliferation, and differentiation all of which are essential processes for tissue regeneration and repair [10-12].

Previous in vitro investigations regarding the regeneration process in dental pulp, HA scaffolds have been studied. They have demonstrated encouraging role of HA in stimulating the mineralization and differentiation of dental pulp stem cells into odontoblastic-like cells [13-15]. According to a different study, HA supports the early formation of dentin and pulp [16]. HA acts as a system for delivery the growth factors since it releases different pro-angiogenic and chemotactic growth factors continuously [14].

Regarded as essential for pulp repair and regeneration are growth factors which are convoluted in diverse cellular activities including neurogenesis, angiogenesis, and cell migration[17]. One of these growth factors used in tissue regeneration investigational studies is vascular endothelial growth factor (VEGF), which is a protein that enhances angiogenesis [18]. It has been proposed to utilize angiogenic-inducing mediators to accelerate the angiogenesis activity in pulp. Essentially, angiogenesis is a crucial stage in the healing from pulpal injuries because sufficient blood flow is needed for tissue regeneration [19]. Previous studies have shown that VEGF may affect tooth development and dentin formation, in addition to encourage the stem cells differentiation into endothelial cells [20,21]. In order to increase our understanding of the role of HA hydrogel in pulp regeneration, we induced pulp exposure and observed the effect of HA on the pulp tissue of incisor teeth. We utilized VEGF as a marker to highlight the regeneration process since it has been shown to up-regulate angiogenesis during tissue regeneration.

Materials and Methods

Study samples and location

In the current study, ten healthy rabbits weighing 1.5 to 2 kg and aged 10 to 12 months were chosen. Each set of animals had its own cage at the Baghdad College of Veterinary Medicine with standard ventilation, food, and housing. They were also provided a consistent diet (pellet and berseem) and allowed full access to water. The animals were randomly divided into two equal groups *(*5 rabbits for each). Twenty teeth total from ten different rabbits were chosen to induce pulp exposure.

Materials and surgical process

The surgical treatment was carried out using a gentle operating approach and in a well-sterilized environment. The weight of each animal was used to determine how much general anesthetic was administered to it. To produce general anesthesia, intramuscular injections of 2% concentration of xylazine and ketamine HCL 50 mg were both administered.

The micro engine's handpiece and 2 mm surgical b ur were established for procedure.

Under aseptic settings, the pulp chamber was opened using a round bur and alternating drilling while being irrigated with normal sterile saline. In the experimental group, a guide hole was drilled in the right upper central incisors until the pulp was reached, following the coronal portion was removed and the pulp exposed. Normal saline irrigation was then used to establish hemostasis, and the cavity was then gently dried with cotton. Upon bleeding control, 0.1 ml of hyaluronic acid gel (HyaDent BG (20 mg Na-hyaluronate/1 ml hyaDent BG) was injected into the pulp chamber with a micropipette and the teeth were temporarily filed to seal them. For the left upper central incisors, the same procedure was carried out, but without the use of hyaluronic acid, and was considered as a control group.

Histological and immunohistochemical method

The animals were killed at the end of each interval (1^{st} week, 2^{nd} week) using an overdose of anesthetic solution, and the tissues were then prepared for histological investigation and immunohistochemical analysis. The fixation of tissue samples were done by of neutral formalin (10%) and processed in standard paraffin blocks. The paraffin-embedded blocks of all of the studied samples were cut into slices of 4 µm thickness and positioned on glass slides for H&E staining. Other 4 µm thick slices were put on positively charged slides to be immunohistochemically localized of VEGF.

The IHC assay procedure was performed following the manufacturer's instructions of monoclonal antibody (VEGF) from Abcam company (ab28775), The cellular localization of (VEGF) is secreted, and mouse specific HRP-DAB detection kit from Abcam company (ab64259) was used for immunohistochemical detection method.

Histomorphometric and immunohistochemical analysis

By considering the area between the pulp's odontoblastic cell layers and the dentin's border, ImageJ software was used to calculate the predentin's thickness [22]. According to the following, the inflammatory intensity was scored: Scores are 1) no or few number of inflammatory cells, 2) Mild (the inflammatory cells fewer than 10 cells), 3) Moderate (the inflammatory cells between 10-25 cells), and 4) Severe (the inflammatory cells further than 25 cells) [23]. The immunohistochemical examination of (VEGF) was done under the light microscope at high power (x400). The mean value of +ve cells (fibroblasts, odontoblasts, and endothelial cells) was calculated [24]. Two calibrated operators performed the to verify the validity of the research.

Statistical analysis

The data were examined using SPSS software, version 25. The mean, median, standard deviation and T-test were used. P values less than 0.05 were regarded as significant

Results

Histological findings

1st week duration

After one week from pulp exposure, the pulp tissue in the control group exhibited disorganization in pulp tissue with blood vessels and necrosis as shown in (Figure 1). Regarding the experimental group showed disorganized pulp tissue, inflammatory reaction, congested blood vessels, and formation of reparative dentin (Figure 2).

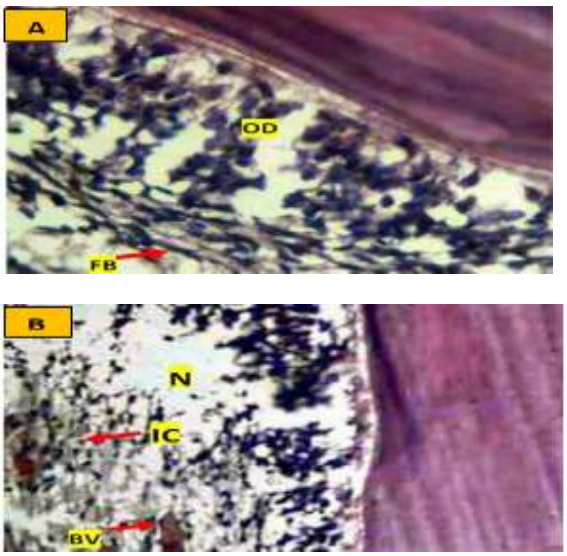

Figure 1. Microscopic picture of pulp tissue in control group after 1week shows (A) disoriented odontoblast cell (OD) and fibroblast(FB)(B) blood vessels (BV), necrotic area (N) and inflammatory cells (IC). H&E (X400).

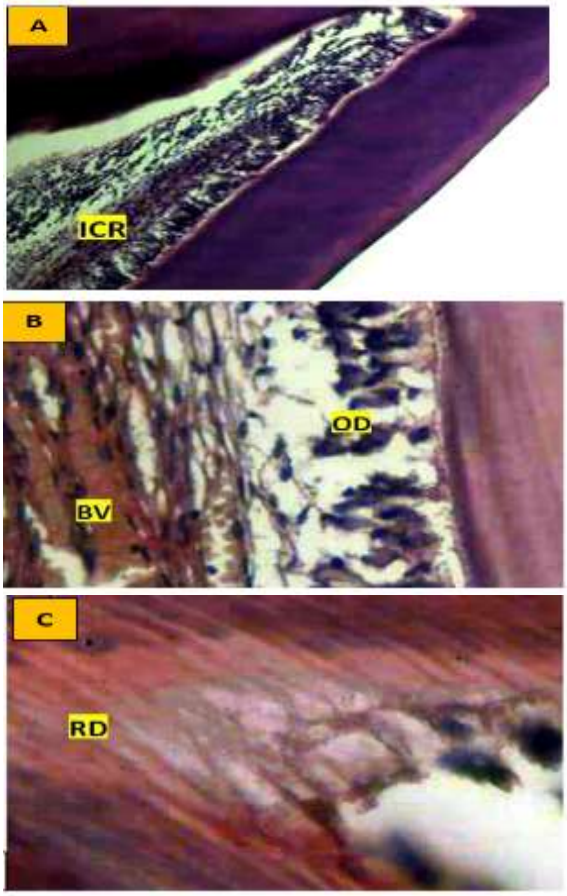

Figure 2. Microscopic picture of pulp tissue in experimental group after 1week shows (A) inflammatory cells reaction (ICR)(B) congested blood vessels (BV) disoriented odontoblast cell (OD) (C) reparative dentin (RD). H&E (X10), (X40) and (X100).

2nd week duration

After two weeks from pulp exposure, the pulp tissue in the control group presented with disorganization in pulp tissue, congested blood vessels, inflammatory reaction, and necrosis (Figure 3). The pulp tissue of the experimental group after two weeks from pulp exposure showed differentiated palisaded odontoblasts, globular mineralization, formation of new blood vessels, a thick layer of predentin, odontoblast-like cells, and formation of osteodentin (Figure 4).

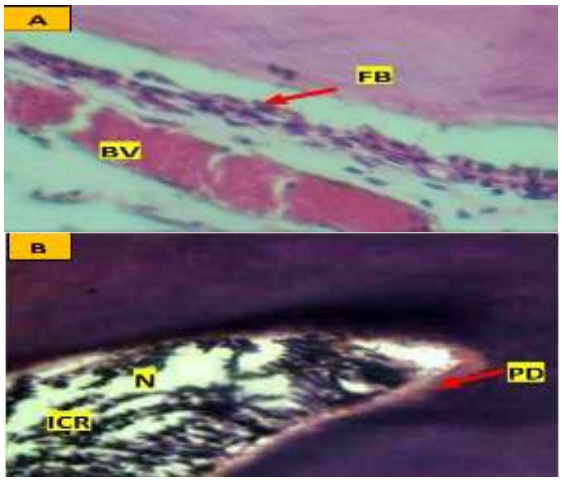

Figure 3. Microscopic picture of pulp tissue in control group after 2week shows (A) fibroblast(FB) and congested blood vessels (BV)(B) necrotic area (N), inflammatory cell reaction (ICR) and predentin(PD). H&E(X40) and (X10).

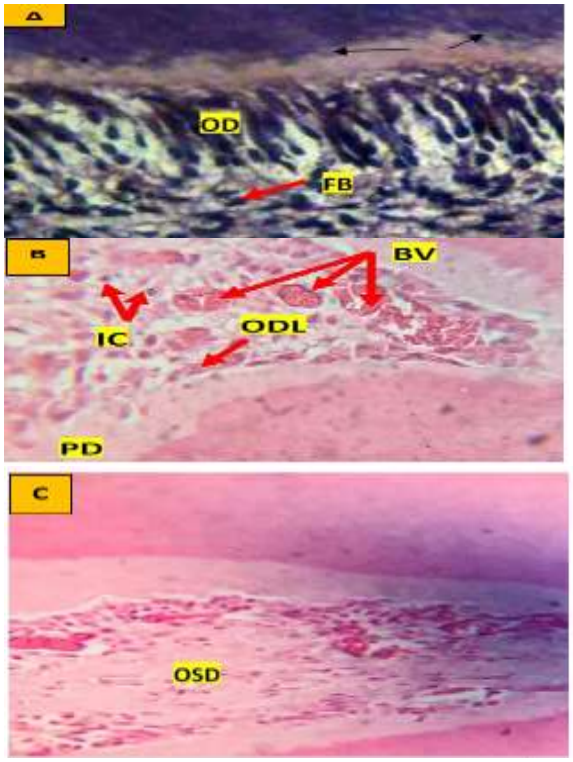

Figure 4. Microscopic picture of pulp tissue in experimental group after 2weeks shows (A) Palisaded odontoblast (OD), fibroblast cells (FB) and globular mineralization(arrows) (B) multiple blood vessels (BV), thick layer of predentin (PD), inflammatory cells (IC) and odontoblast like cells (ODL)(C) osteodentin (OSD). H&E (X400).

Immunohistochemical findings

1st week duration

After one week duration, the control group showed mild immunoreactivity to VEGF mostly in disorganized odontoblast cells and predentin (Figure 5). The experimental group in 1st week duration showed moderate positive immunoreactivity to VEGF mostly in endothelial cells as described in (Figure 6).

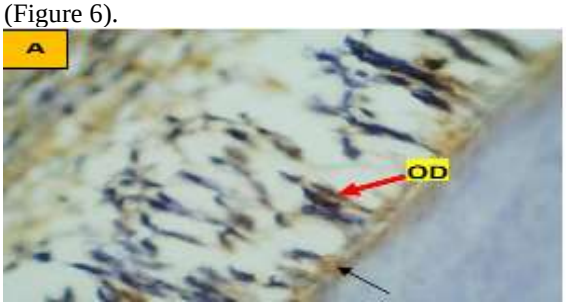

Figure 5. Immunohistochemical picture of control group in 1st week shows positive expression in odontoblasts(OD) and predentin(Black arrow). DAB stain with hematoxylin counter stain X400.

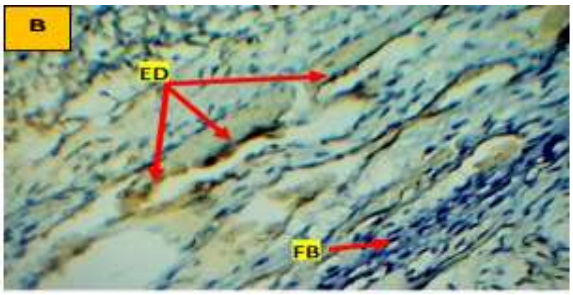

Figure 6. Immunohistochemical picture of experimental group in 1st week shows strong positive expression in endothelial cells (ED) and negative expression in fibroblast (FB). DAB stain with hematoxylin counter stain X400.

2nd week duration

In the 2nd week duration the control group also showed mild immunoreactivity to VEGF in fibroblast cells and weak expression in odontoblasts (Figure 7). While in the experimental group the pulp tissue in 2nd week duration showed strong expression of VEGF in endothelial cells, odontoblasts, fibroblasts, predentin, and collagen fibers (Figure 8).

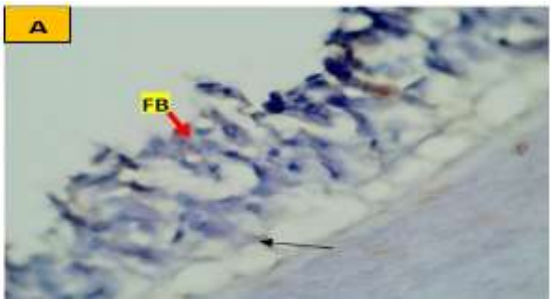

Figure 7. Immunohistochemical picture of control group in 2nd week after pulp exposure shows positive expression in fibroblast cells(FB) and weak expression in odontoblasts(Black arrow) DAB stain with hematoxylin counter stain X400.

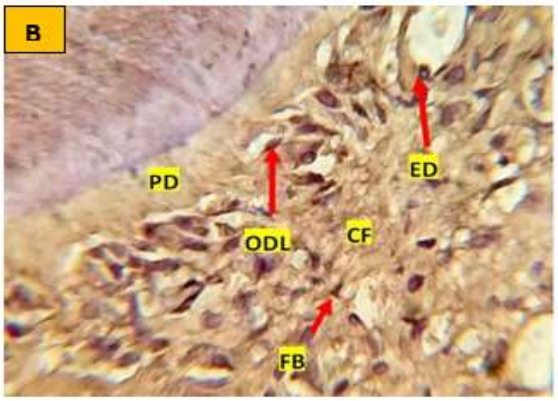

Figure 8. Immunohistochemical picture of experimental group in 2nd week duration shows strong positive expression in odontoblast(ODL), fibroblast (FB) endothelial cells (ED),collagen fibers (CF), and predentin (PD) DAB stain with hematoxylin counter stain X400.

Statistical results

In (Table 1.) illustrates comparison differences in inflammatory intensity between the control and experimental group in each period. In both periods, There were no statistically significant differences among groups with a high median value of inflammatory intensity in the experimental group in 1st week duration, The control group was regarded as having a high median value of inflammatory intensity in the 2nd week duration as shown in (Figure 9).

Table 1. Comparative difference of inflammatory intensity between all groups in each period

Groups	median	Chi-square	P value
Control group 1st week	2	1.0664	.899568 NS
Experimental group 1st week	4		
Control group 2nd week	3	0.625	.960246 NS
Experimental group 2nd week	2		

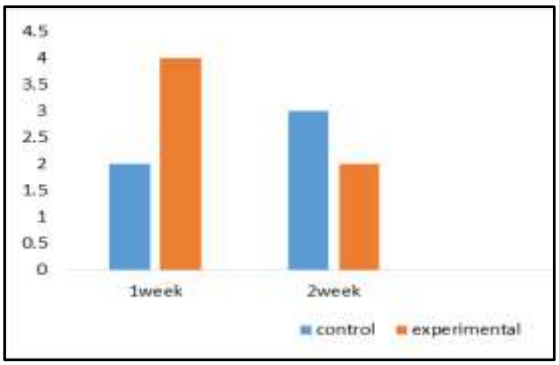

Figure 9.Comparisons in the mean value of inflammatory intensity among groups at each period.

Table 2. shows the comparison between the control and experimental group by using T-test for predentin thickness. The result presented non-significant differences at 1st week duration and significant differences at 2nd week duration with the highest mean value of predentin thickness in the experimental group at 2nd week duration (Figure 10).

Table 2 . Comparison difference of predentin thickness between all groups in each period using T-test.

Groups	Mean±SE	*t*-value	P value
Control group 1st week	5.7±1.12	0.96873	.180527 NS
Experimental group 1st week	7.5±1.33		
Control group 2nd week	16.6±3.8	7.53817	.000033 S
Experimental group 2nd week	56.3±4.2		

SE: Standard error

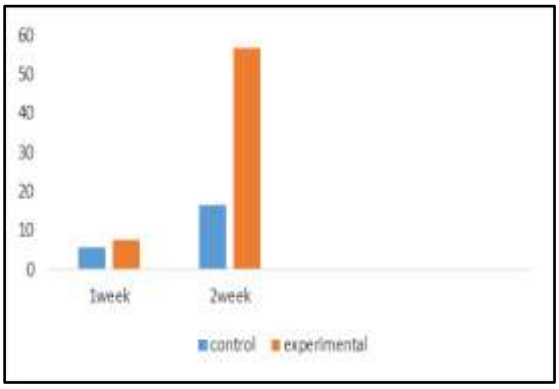

Figure10. mean values of predentin thickness measurement in all groups and at each study period.

Table 3. reveals the group comparison differences in immunoreactivity to VEGF antibody in pulpal cells. The experimental group had the highest mean value of positively expressed pulpal cells during the 2nd week duration, as shown in (Figure11), and the results revealed significant differences between the control and experimental groups throughout both periods.

Table 3.Group comparison differences in immunoreactivty to VEGF in pulpal cells using T-test.

Groups	Mean±SE	*t*-value	P value
Control group 1st week	8.9±1.4	12.21019	**.00001 S**
Experimental group 1st week	22.9±1.7		
Control group 2nd week	11.9±0.95	-8.10707	.00002 **S**
Experimental group 2nd week	34.3±1.9		

SE: Standard error

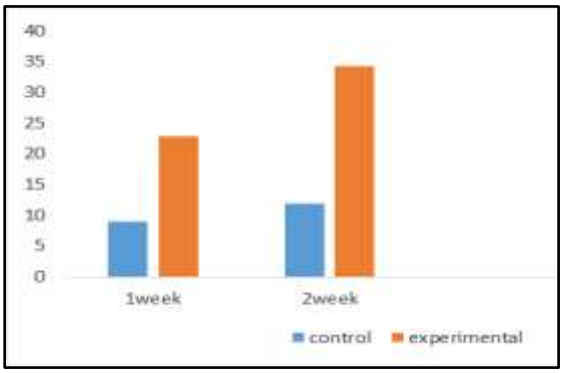

Figure11. mean value of positively expressed pulpal cells in all groups in both periods.

Discussion

Specific properties should be available in the materials in order to be a suitable scaffold include that the material should be biodegradable, capable of stimulating infiltration of different cells, vascularization, and induce differentiation of cells, and ultimately be replaced by the healthy tissues [25]. Because of its biocompatibility, biodegradation capabilities, and most significantly its function in the development of dental tissue, The use of HA gel as a potential scaffold for dental pulp regeneration has been suggested [26].

Angiogenesis is a crucial stage in the effective recovery of injured pulp. The richly vascularized tissue of the pulp provides defense against repeated inflammatory insults [27]. Angiogenesis, which occurs before the formation of reparative dentine, is assisted by the release of angiogenic growth factors through pulp cells caused by damage [28]. Angiogenesis is essential for the successful repair, healing, and regeneration of tissues; without suitable blood flow, the regeneration of tissue is challenging and scar or necrotic tissues result as a consequence [29]. The most effective angiogenic agent involved in the production of tertiary dentin is VEGF which is a protein released specifically by endothelium, is crucial for angiogenesis and pulp regeneration [30].

Therefore, the aim of the study was to investigate the hyaluronic acid's ability for regeneration in tooth pulp tissue using histological analysis and immunohistochemical localization of VEGF in pulp tissue. Typically, the dental pulp is exposed to a variety of irritants, which might have an impact on the pulp's function and health. Each form of irritant has a particular effect on the pulp, including cavity preparation, dental caries, traumatic disturbances, and chemical compounds. These factors could be responsible for the inflammation's onset [31,32].

There is evidence that inflammation is essential for tissue healing as a first stage, after which the pulp is regenerated. The healing process begins with an initial mild inflammatory response [33]. These details provided an explanation for the study's findings, which showed that there was no significant difference in the intensity of the inflammatory response among the control and experimental groups within the first week following pulp exposure. The present findings showed that in 2nd week duration, a reduction in the inflammatory response was evident in the experimental group while regarding the control group there was persistent inflammatory response and necrosis. This suggested a positive healing outcome mediated by hyaluronic acid, because previous study carried by Dahiya (2013)[34] who revealed the roles of hyaluronic acid in a number of biological processes, such as anti-inflammatory action and enhancing hard and soft tissue healing responses.

Additionally, another previous studies have verified that the interaction of cells in pulp tissue with HA can cause stem cells differentiation into odontoblast like cells and stimulate the production reparative dentin. As a result, HA gel might be appropriate for pulp regeneration therapies[15,26] and this was consistent with the current histological findings, which showed the formation of odontoblast like cells and reparative dentin in the experimental group in both periods. The present study revealed an increase in predentin thickness in the experimental group in both periods with significantly different from the control group, indicating active dentinogenesis. These findings are in accordance with those of other research that point to the function of HA in the mineralization and dentin formation processes and suggest that it has

the potential to be a good scaffold for dental pulp regeneration [26, 35, 36].

Because of the necessities of nutrition source and waste elimination for the proper effective of the vascularization, it has been demonstrated that the metabolites of HA, which are regularly degraded, stimulate angiogenesis and play a significant part in tissue regeneration [37] and this explained the histological results of the experimental group at 2nd week duration which revealed formation of multiple new blood vessels in pulp tissue. The IHC findings of the current study revealed the maximum mean value of VEGF-positive expression in pulpal cells, especially in endothelial cells and odontoblasts in the pulp tissue of the experimental group with a significant difference as compared the control group in both periods. Therefore, the present study proposed that HA has an active role in angiogenesis as mentioned before, by inducing mesenchymal cell recruitment and differentiation, then produced different growth factors such as VEGF as a cellular reaction.

VEGF promotes endothelial cell proliferation, improves the flow of blood in pulp tissue, and increases the hyperpermeability of capillaries [38]. Endothelial cells represent the main sources of VEGF secretion in response to injury [28]. Odontoblasts also express VEGF [39], trauma, orthodontic pressures, and hypoxia can all influence its expression [40]. Because the matrix of dentin includes angiogenic growth factors and are produced after injuries to promote reparative responses in dentin- pulp complex [41]. This explained the IHC results which exhibited strong positive labeling to VEGF in predentin frequently in the experimental group in 2nd week duration.

According to a previous study, VEGF expression is different in reversible pulpitis which may indicate that this factor, may have a protective function in the early stages of inflammation and have the capacity to promote pulp angiogenesis and healing [42]. Furthermore, according to earlier study [43], it has been established that VEGF promotes the cells of human pulp to proliferate and differentiate into odontoblasts. These data imply that VEGF could be a helpful growth factor for repairing pulp and dentin injury [44]. When considered collectively, to hypothesize that VEGF, in addition to its primary angiogenic function, may also have a direct effect on odontoblast development and activity.

Conclusion

The results of this study provide an encouraging illustration of improved pulp regeneration that occurs after the application of HA gel through angiogenesis, odontoblastic-like differentiation, and reparative dentin formation. This was demonstrated by an increase in the expression of VEGF throughout the pulp tissue cells. Further research is mandatory to determine the possible clinical application of HA gel in regenerative endodontics.

Acknowledgment

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Conflicts of Interest

None.

Funding

None.

Ethics Statement

The study was approved by the College of Dentistry / University of Baghdad's local ethics commission (project No. 386721, Ref. number: 386).

Author Contribution

Conceptualization, RJ, NB and AW; methodology, NM and BA; software, NB and AW; validation, RJ, NB, and NM; formal analysis, RJ; investigation, NM and AW; resources, RJ; data curation, NB and AW; writing original draft preparation, RJ; writing, review and editing, RJ and NB; visualization, BA and AW; supervision, BA and NM; project administration, RJ. The available form of the manuscript has been revised and accepted by all authors.

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