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Enhancement of MTA Repair HP with Hyaluronic Acid and TGFβ1 in Rabbit Pulpotomy

A Chemical-Histological and Immunihistochemical Evaluation

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Abstract

Vital pulp therapy (VPT) preserves the pulp's vitality, enabling its defense and reparative functions. This is a well-known property of mineral trioxide aggregate (MTA), but the use of bioactive modifiers can be added to improve healing. There are known factors that affect inflammation, odontoblastic differentiation and tissue regeneration such as hyaluronic acid (HA) and transforming growth factor beta 1 (TGFβ1). The objective of this work was to compare histological and immunohistochemical reactions of the pulp in the MTA Repair HP modified with HA and/or TGF-β1 in a pulpotomy rabbit model. Methods: 9 male albino rabbits, Oryctolagus cuniculus were subjected to pulpotomies in four incisors, which were randomized to MTA (control), MTA+HA, MTA+TGFβ1 and MTA+HA+TGFβ1 groups. At 1, 3 and 7 days, the animals were sacrificed. Histological evaluation was done to evaluate inflammatory cell infiltration, cellular organization and odontoblast-like cell formation. The expression of TGFβ1 was analyzed by immunohistochemistry. Nonparametric tests were used ($p \leq 0.05$). There was the least inflammation in MTA+HA+TGFβ1 group at day 7, and the most organized pulpal architecture: aligned odontoblast-like cells and dense collagen fibrils ($p<0.05$ vs. control). The cell formation of odontoblasts was the highest in the group treated with TGFβ1, especially in the HA co-treatment group. The MTA+HA+TGFβ1 group expressed the highest level of TGFβ1 at all time points, and it was strongly expressed in odontoblast-like cells, fibroblasts and endothelial cells. HA and TGFβ1 when combined in MTA Repair HP, synergistically increased early pulp healing through minimizing inflammation, enhancing tissue organization and upregulating the expression of TGFβ1, suggesting its potential as a biologically based modification for VPT.

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Introduction

VPT is a biologically based clinical approach that aims to maintain the vitality and function of the dentinal pulp after mechanical or carious exposure, thus preserving the intrinsic defensive, reparative and supportive properties of the dentinal pulp to maintain dentinogenesis. The maintenance of pulp vitality prevents the need for more aggressive root canal treatment, preserves proprioceptive feedback and pulp-mediated immune defense mechanisms in the dentin-pulp complex [1,2].

Mineral trioxide aggregate (MTA) is considered a gold standard biomaterial for VPT because of its biocompatibility, bioactivity, sealing capacity and proven capacity to stimulate hard tissue barrier formation [3,4].

In addition, MTA actively contributes to the healing by releasing calcium ions that induce odontoblastic activity and mineralized tissue deposition (5). However, there has been a recent increase in interest in more effectively leveraging its biological effect by adding bioactive adjuncts that are able to alter the local tissue microenvironment. Hyaluronic acid (HA) is a large molecular weight glycosaminoglycan that is important for wound healing and tissue remodeling, acting to regulate inflammation, stimulate proliferation of fibroblasts and promote ECM organization [6,7]. A multifunctional cytokine that is plentiful in the dentin matrix, transforming growth factor beta 1 (TGFβ1), has

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been shown to regulate odontoblast differentiation, promote recruitment of dental pulp stem cells, and stimulate tertiary dentinogenesis [8,9].

The individual attributes of HA and TGFβ1 in pulp healing have been proven [10], but little evidence has been available for their application together in a MTA-based paste, and whether the biological synergy between them can enhance the resolution of inflammation, odontoblast-like cell differentiation and quality of regenerated pulp tissue. In the present study, the authors investigated the histological and immunohistochemical responses of the pulp tissue after MTA Repair HP modified with HA and TGFβ1 in a rabbit vital pulpotomy model with special focus on inflammatory modulation, cellular organization, and the pattern of expression of TGFβ1 during the early healing phase, in order to address this gap in knowledge.

Material and Methods

Nine healthy, mature male albino rabbits (*Oryctolagus cuniculus*), aged 9–12 months and weighing 1.0–1.7 kg, were housed under controlled environmental conditions with ad libitum access to food and water. Ethical approval was obtained from the Research Ethics Committee, College of Dentistry, University of Mosul (REC No. UoM.Dent.25/1016).

Preparation of Modified MTA Repair HP Formulations: Recombinant human TGF-β1 protein (10µg) (Elabscience Biotechnology Inc., USA) is delivered as a sterile white lyophilized (freeze-dried) powder that must be reconstituted before use. According to the manufacturer's instructions, the vial was centrifuged before opening. Then, the lyophilized powder was diluted with pyogenic-free distilled water to obtain a stock solution. So, (1ml) of pyogenic-free distilled water was added to the lyophilized powder. Then, from the stock solution, 20 µl was aspirated using a micropipette and further diluted by adding 39.98ml of pyogenic-free distilled water until obtaining a solution (40ml) in a concentration of (1ng/200µl) TGFβ1 [11]. Each (1ml) was put in an Eppendorf tube and stored in a tube rack at -20°C for future use.

The MTA powder is supplied as capsules, each of (0.085 g). By following the manufacturer's instructions, 0.085 g of powder (1 capsule) was mixed with 34 µl of liquid [12]. All experimental groups adhered to the same ratio of MTA powder to mixing liquid. The mixed liquids were: 1- The distilled water provided by the MTA manufacturer, 2- HMW-HA, 3- The distilled water in which TFGB1 is diluted, and 4- The mixed liquid that contains HMW-HA and TFGB1. A metal spatula is employed for mixing using a glass slab to create a paste with a putty-like consistency. The groups studied were:

1. Control group: Unmodified MTA Repair HP.
2. MTA + HA: MTA Repair HP powder with (34 µl) HA.
3. MTA + TGFβ1: MTA Repair HP powder with (1ng /200µl) TGFβ1.
4. MTA + HA + TGFβ1: MTA Repair HP powder with both 20 mg/ml HA and (1ng /200µl) TGFβ1.

All materials were prepared under aseptic conditions immediately before placement to avoid premature setting and to preserve the bioactivity of the incorporated agents.

General anesthesia was induced via intramuscular injection of ketamine hydrochloride (50 mg/kg; Alfasan, Holland) and xylazine hydrochloride (5 mg/kg; Interchemie, Holland). Following aseptic preparation and rubber dam isolation, access cavities were prepared on the labial surface of maxillary incisors and lingual surface of mandibular incisors using sterile round carbide burs under copious sterile saline irrigation. The pulp was exposed (~1 mm diameter), bleeding was controlled with sterile cotton pellets moistened in 0.9% NaCl, and the assigned pulp capping material was applied immediately. Cavities were sealed with a chemically cured glass ionomer and light-cured flowable composite resin.

After euthanasia by overdose of ketamine/xylazine, treated teeth were extracted, fixed in 10% neutral buffered formalin, decalcified, processed, and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H&E) for assessment of inflammatory cell infiltration, cellular organization, and odontoblast-like cell formation.

Scoring followed a non-parametric ordinal scale, with evaluation performed by blinded observers.

TGFβ1 expression was evaluated using a rabbit-specific polyclonal anti-TGFβ1 antibody. Sections underwent antigen retrieval, peroxidase blocking, incubation with the primary antibody, and visualization with a DAB chromogen. Expression was semi-quantitatively scored (0–4) based on staining intensity and distribution within odontoblasts, fibroblasts, endothelial cells, and pulp stroma.

Data were analyzed using IBM SPSS Statistics v23 (IBM Corp., USA). Non-parametric comparisons among groups at each timepoint were performed using the Kruskal–Wallis test, followed by Dunn's post hoc test for pairwise comparisons. Within-group temporal changes were analyzed.

Results

Median inflammatory scores across timepoints are summarized in Table 1. At day 1, all groups exhibited moderate to severe inflammation, with the highest scores in the control group. A progressive reduction in inflammation was observed in all groups over time, with the MTA+HA+TGFβ1 group showing the most marked decrease by day 7 ($p < 0.05$ compared to control).

As can be seen in Figure 1, the organization of cells was better with the passage of time in all the groups, the most organized pulpal architecture being present in the combination group at day 7. This included the presence of oriented odontoblast-like cells, low interstitial oedema and abundant collagen fibrils.

On day 7, the highest mean and median scores for odontoblast-like cell formation were observed in the groups that received TGFβ1, with no differences between the combination and other groups that received TGFβ1 (Table 2). The cells were elongated with processes extending towards the dentin and had polarized nuclei, suggesting an active reparative dentinogenesis.

Histological micrographs (H&E staining, 100× and 400× magnification) showing pulp tissue responses at day 7 in all experimental groups. Control MTA group exhibited mild inflammatory infiltrate with limited fibrous tissue formation (Figure 2); MTA+HA group showed moderate fibroblast proliferation and early mesenchymal cell recruitment (Figure 3); MTA+TGFβ1 group revealed well-developed fibrous tissue and organized odontoblast-like cells (Figure 4); MTA+HA+TGFβ1 group displayed the highest organization with dense collagen bundles, minimal edema, and intense odontoblastic alignment (Figure 5).

Day 7 section from the MTA+HA+TGFβ1 group showing dense collagen bundles (arrow), minimal edema, and intense odontoblastic alignment (arrowhead), consistent with the highest median cellular organization score in Table 2.

TGFβ1 immunoexpression was highest in the MTA+HA+TGFβ1 group at all timepoints, particularly at day 3 (Table 3). Staining was prominent in odontoblast-like cells, fibroblasts, and endothelial cells, indicating both reparative and angiogenic activity. The control group showed minimal expression at day 1 with gradual increase by day 7 but remained significantly lower than the experimental groups ($p < 0.05$). Quantitative image analysis of TGFβ1 staining using ImageJ software is recommended for future studies to complement semi-quantitative scoring and reduce observer bias.

Immunohistochemical micrographs (TGFβ1 expression, 100× magnification) of rabbit pulp tissue at days 1, 3, and 7. Control MTA group showed mild to moderate expression over time (Figure 6); MTA+HA group demonstrated progressive increase in positive staining (Figure 7); MTA+TGFβ1 group exhibited strong, sustained

expression (Figure 8); MTA+HA+TGFβ1 group revealed intense expression at all time points, indicating synergistic upregulation of TGFβ1 in pulp regeneration (Figure 8).

Discussion

The present study demonstrated that the incorporation of hyaluronic acid (HA) and transforming growth factor beta 1 (TGFβ1) into MTA Repair HP markedly enhanced the pulpal healing response following vital pulpotomy in a rabbit model. The percentage of inflammatory cell infiltration showed a gradual decrease and the grade of cellular organization showed a gradual improvement throughout the 7 days of evaluation, being the combination group (MTA+HA+TGFβ1) the most effective group. The decrease in inflammatory response in HA containing groups may be explained by the well-known anti-inflammatory and wound healing effects of HA, which act by inhibiting free radicals, stimulating angiogenesis, and supporting cell migration [13,14].

TGFβ1, however, is an important factor regulating the differentiation of odontoblasts and the deposition of extracellular matrix, directly stimulating reparative dentinogenesis [15,16]. The combined group showed a synergistic effect in favor of the hypothesis that two different modulations of the inflammatory environment (HA) and stimulation of the odontogenic signals (TGFβ1) can accelerate the regeneration of pulp tissues. Notably, the formation of odontoblast-like cells was significantly higher in TGFβ1-treated groups, consistent with previous reports highlighting the role of this cytokine in upregulating dentin matrix proteins such as dentin sialophosphoprotein (DSPP) and osteocalcin [17]. These cells were organized, as well as the dense collagen matrix observed histologically, suggest that the regenerated tissue was oriented in a functional way towards the repair of dentin, rather than fibrotic healing.

This was further supported by immunohistochemical analysis which showed significant increase in expression of TGFβ1 in experimental groups particularly combination group at day 3. This peak expression is probably from an early reparative stage with active signaling for cell proliferation, differentiation and angiogenesis. In comparable temporal expression models of pulp healing early expression of TGFβ1 is associated with good late results [18,19]. Overall, the data suggest that modifying MTA Repair HP with HA and TGFβ1 can modulate the early biological events following pulpotomy, leading to faster resolution of inflammation, enhanced odontoblast differentiation, and improved pulp tissue organization. These responses could be of clinical importance in cases of accelerated healing, or even in vital pulp therapy.

The present results show obvious histological and immunohistochemical benefits from the combined HA and TGFβ1 modification; however, the follow-up period is relatively short (7 days) and limits the extrapolation to long-term effects of dentin bridge maturation and functional restoration of the pulp. Furthermore, the limited number of subjects (n=3/time point) may have limited the statistical power to detect small differences between groups. To support and validate the present findings, further studies are recommended with larger sample size, longer observation period and quantitative morphometric and immunohistochemical examination. The increased inflammatory modulation and organization of odontoblast-like cells in the HA- and TGFβ1-modified groups agree with previous experimental studies showing pulp healing properties of HA in inflammatory models [6,13]. In the same way, the generation of more odontoblast-like cells and elevation of the expression of TGFβ1 in the current study agree with previous studies of the exogenous addition of TGFβ1 to dental pulp stem cell differentiation and stimulation of tertiary dentin deposition [9,17]. Results from the MTA+HA+TGFβ1 group showed that the synergistic effect is greater than the regeneration achieved by either agent alone in previous studies, indicating that simultaneous regulation of the inflammatory microenvironment and activation of odontogenic signaling pathways may lead to additive or even multiplicative effects. This is consistent with the principles of tissue engineering which state that using multiple bioactive molecules with different mechanisms provides building blocks for a more robust, and more functional, repair.

Conclusion

In this in vivo study, within the limitations, the HA incorporation along with the inclusion of TGFβ1 in the MTA Repair HP significantly enhanced the histological and immunohistochemical assessment of pulp healing following vital pulpotomy. The lowest inflammatory score, the most organized cellular architecture and the highest expression of TGFβ1 in the combination group, indicating a synergistic regenerative effect. These results support a strong biological basis for continued preclinical and clinical testing of MTA formulations with bioactive agents for the ability to improve pulp tissue regeneration.

Limitations

The number of experimental animals used per time point in this study is small and may impact the statistical significance of the results. In addition, the lack of physicochemical characterization of the modified MTA formulations prevents any conclusions regarding possible alteration of material properties that might affect handling and setting properties. The short term follow up (7 days) offers some information about early events of pulp healing but is not relevant to long term quality and function of the dentin bridge.

Clinical Implications: The use of HA and TGFβ1 with MTA Repair HP may provide a biologically enhanced pulp capping material that could facilitate pulp healing and enhance the success of vital pulp therapy, especially when early dentinogenesis and rapid inflammation resolution is desired.

Conflict of Interest

The authors declare no conflict of interest.

Funding

None.

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Table 1. Histopathological scores of inflammatory cell response among study groups in rabbits.

Groups / Intervals	Day 1 (N=3) Median (IQR)	Day 3 (N=3) Median (IQR)	Day 7 (N=3) Median (IQR)	P-value
Control (MTA) group	3 (2) A a	2 (1) AB b	1 (2) A c	0.028
MTA + HA group	2 (1) B a	2 (2) AB a	1 (1) A b	0.048
MTA + TGFβ1 group	2 (1) B ab	3 (2) A a	1 (0) A b	0.032
MTA + HA + TGFβ1 group	2 (1) B a	1 (1) B a	0 (0) B c	0.028
P-value	0.044	0.034	0.048	

Notes: Data expressed as Median & IQR (Inter-Quartile Range) for N = 3 rabbits.

- Kruskal–Wallis test was used.
- Capital letters indicate significant differences between groups at $p \leq 0.05$.
- Small letters indicate significant differences between periods at $p \leq 0.05$.

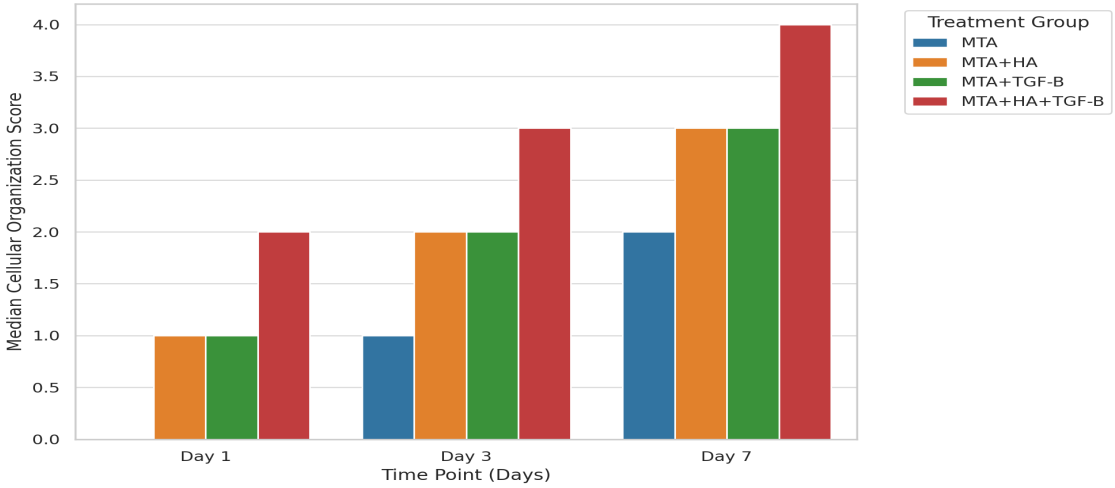


Figure 1. Median cellular organization score over time by treatment group.

Table 2. Histopathological scores of odontoblast-like cell formation among study groups in rabbits.

Groups / Intervals	Day 1 (N=3) Median (IQR)	Day 3 (N=3) Median (IQR)	Day 7 (N=3) Median (IQR)	P-value
Control (MTA) group	0 (0) B b	1 (0) B a	1 (1) C a	0.042
MTA + HA group	1 (1) AB b	2 (1) A a	2 (1) B a	0.044
MTA + TGFβ1 group	1 (1) AB c	2 (1) A b	3 (1) A a	0.034
MTA + HA + TGFβ1 group	2 (1) A b	2 (0) A b	3 (0) A a	0.038
P-value	0.044	0.048	0.028	

Notes:

- Data expressed as Median & IQR (Inter-Quartile Range) for N = 3 rabbits.
- Kruskal–Wallis test was used.
- Capital letters indicate significant differences between groups at $p \leq 0.05$.
- Small letters indicate significant differences between periods at $p \leq 0.05$.

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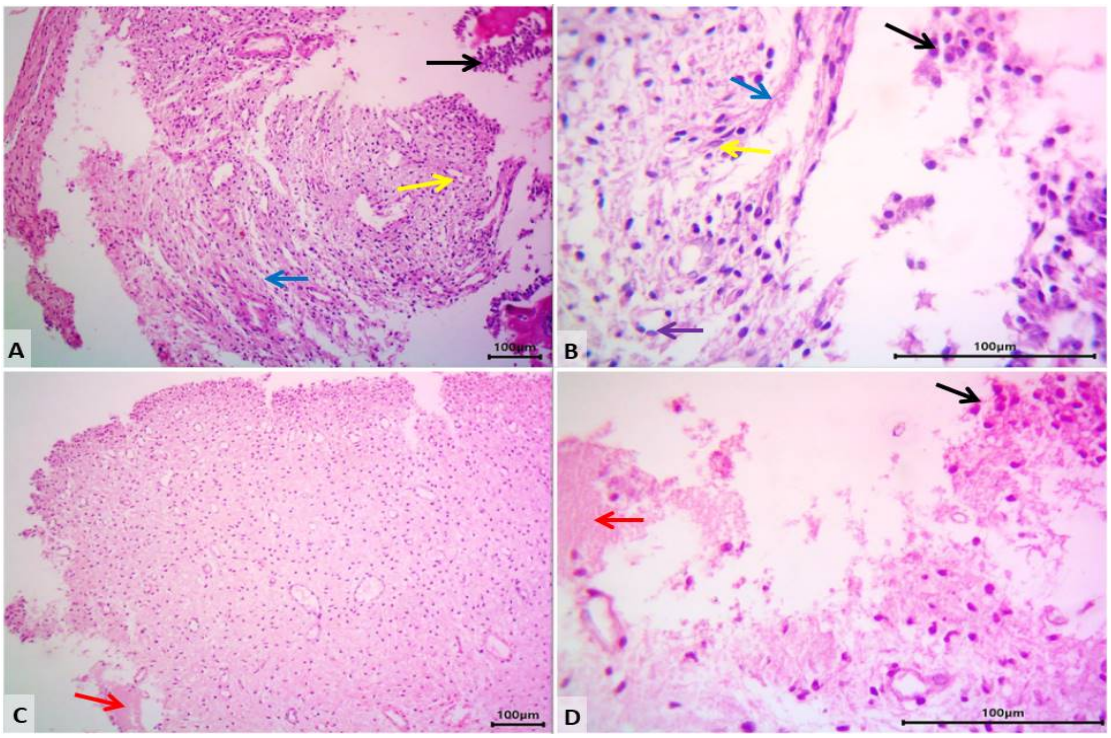


Figure 2. Histological sections of the pulp tissue of rabbit at day 7 post-operation of the (MTA) group shows slight infiltration of inflammatory cells (→), few fibrous tissue (→), edema (→), proliferating cell as fibroblasts (→), endothelial cells (→). Scale-bar=100µm. [A&C]: 100X; [B&D]: 400X.

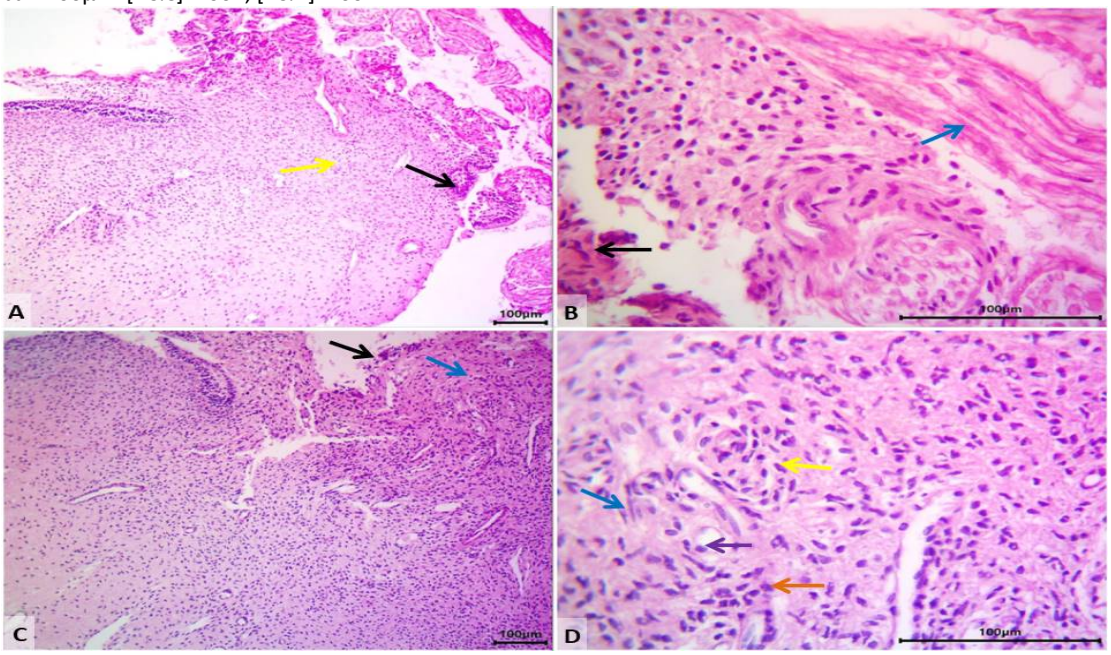


Figure 3. Histological sections of the pulp tissue of rabbit at day 7 post-operation of the (MTA+HA) group shows slight infiltration of inflammatory cells (→), fibrous tissue (→), proliferating cell fibroblasts (→), endothelial cells (→) and mesenchymal cells (→). Scale-bar=100µm. [A&C]: 100X; [B&D]: 400X.

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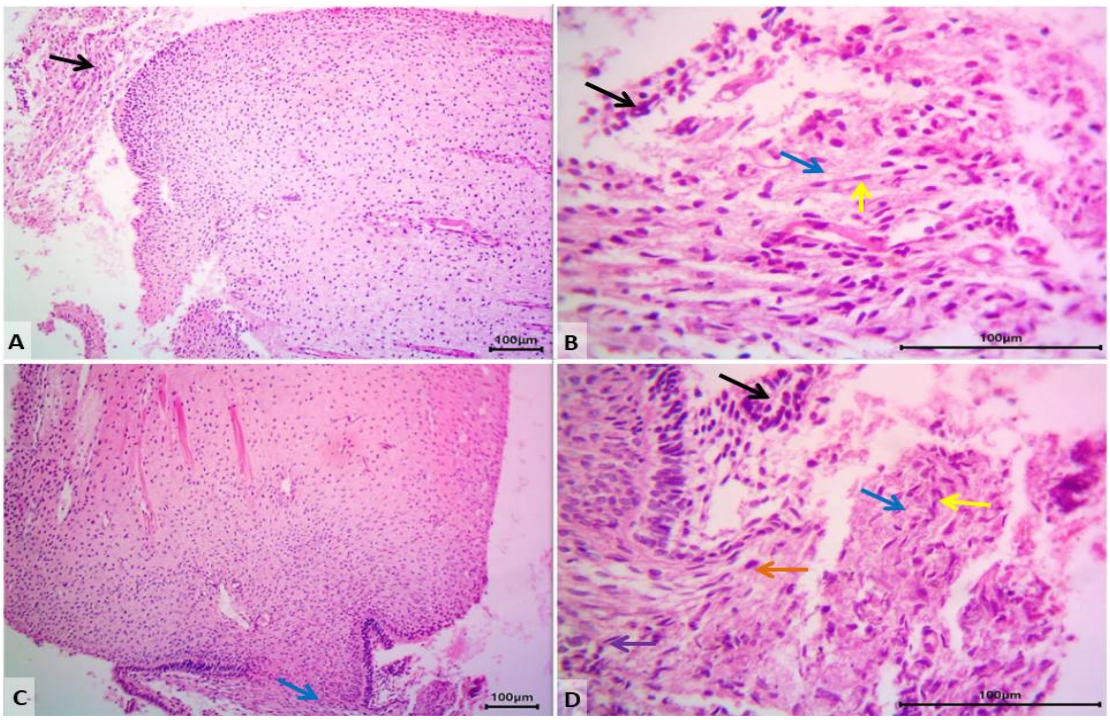


Figure 4. Histological sections of the pulp tissue of rabbit at day 7 post-operation of the (MTA+TGFB1) group shows slight infiltration of inflammatory cells (→), well-developed fibrous tissue (→), proliferating fibroblasts (→), endothelial cells (→) and mesenchymal cells (→). Scale-bar=100µm. [A&C]: 100X; [B&D]: 400X.

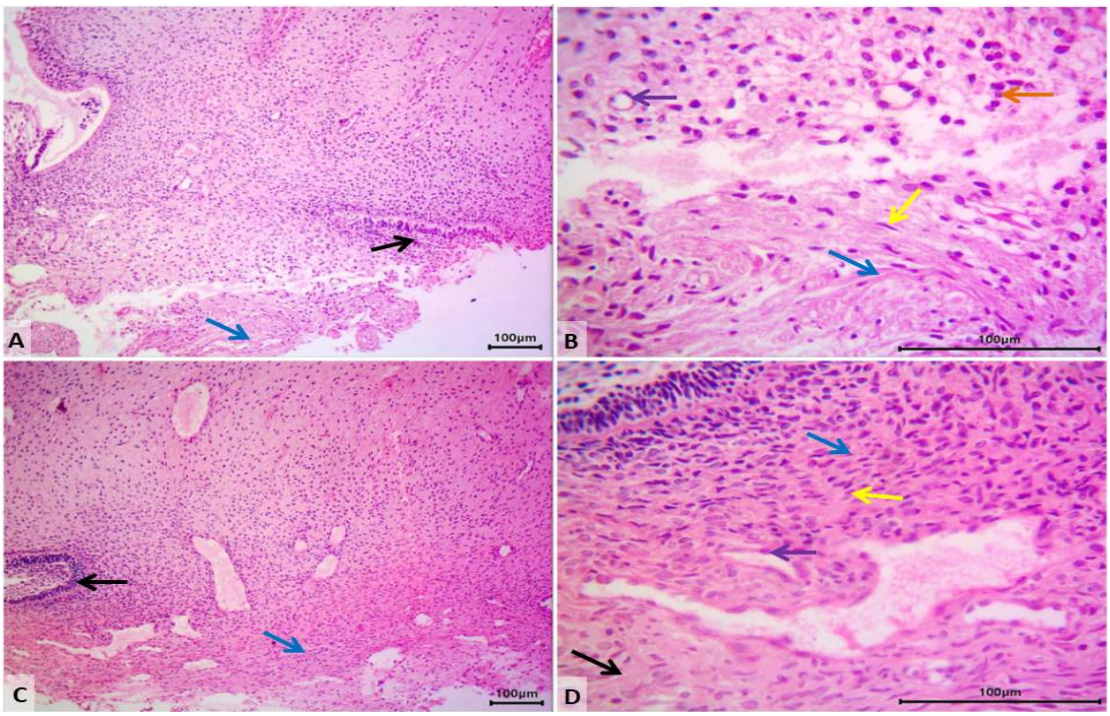


Figure 5. Histological sections of the pulp tissue of rabbit at day 7 post-operation of the (MTA+HA+TGFB1) group shows proliferating odontoblasts (→), well-developed fibrous tissue (→), proliferating fibroblasts (→), endothelial cells (→) and mesenchymal cells (→). Scale-bar=100µm. [A&C]: 100X; [B&D]: 400X.

Table 3. Semi-quantitative TGFβ1 immunoexpression scores (median, IQR) for all groups at each timepoint.

Group	Day 1	Day 3	Day 7
MTA	Slight (+)	Moderate (++)	Mild (++)
MTA+HA	Mild (+)	Mild (+)	Moderate (++)
MTA+TGFβ1	Strong (+++)	Moderate (++)	Strong (+++)
MTA+HA+TGFβ1	Intense (++++)	Intense (++++)	Very Intense (+++++)

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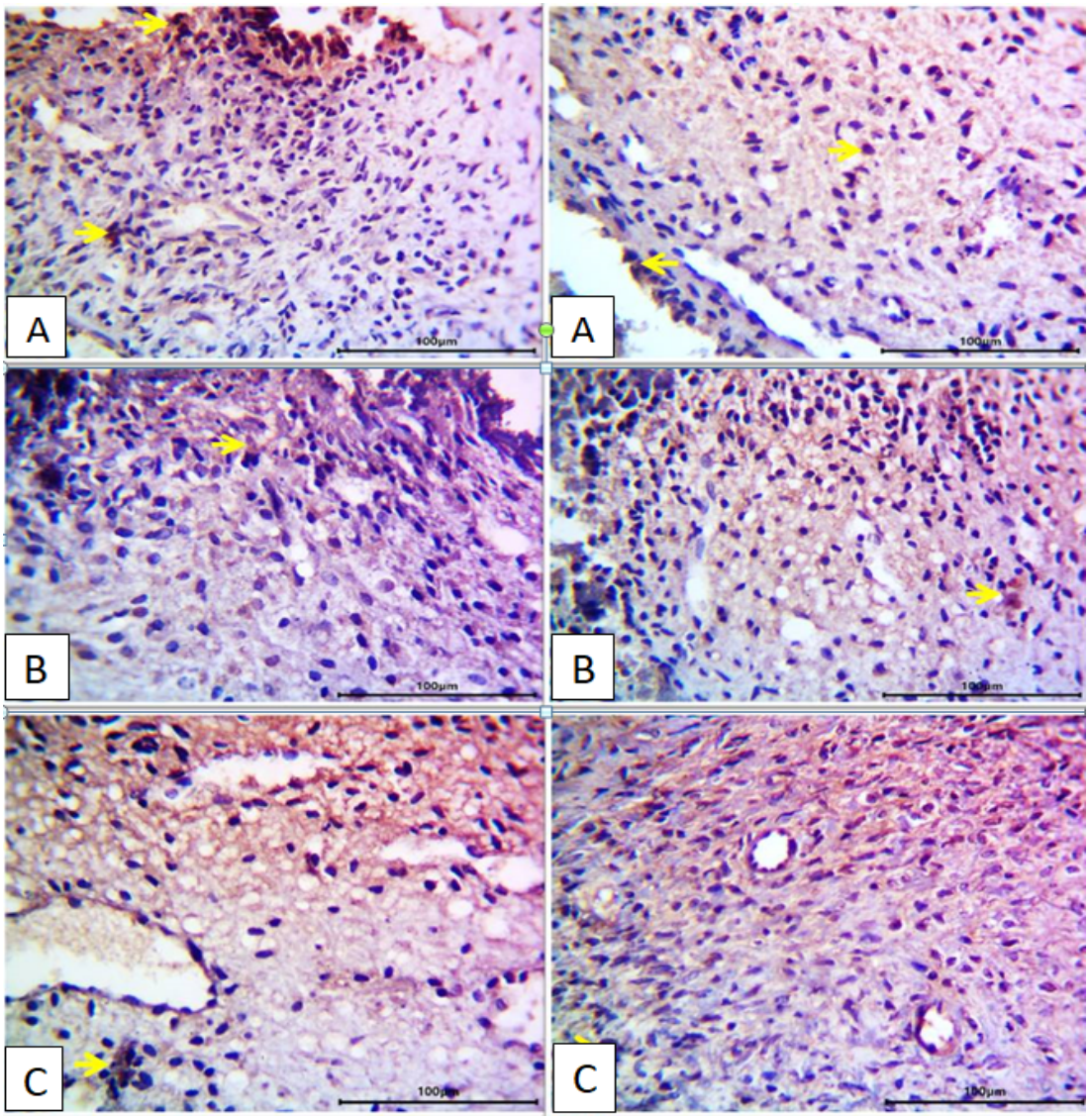


Figure 6. Representative composite photomicrographs of TGFβ1 immunoexpression in pulp tissue for the control (MTA repair HP only) group at days 1(A), 3(B), and 7(C) (×400 magnification). Immunostaining intensity decreased over time from moderate–severe at day 1 to mild at day 7.

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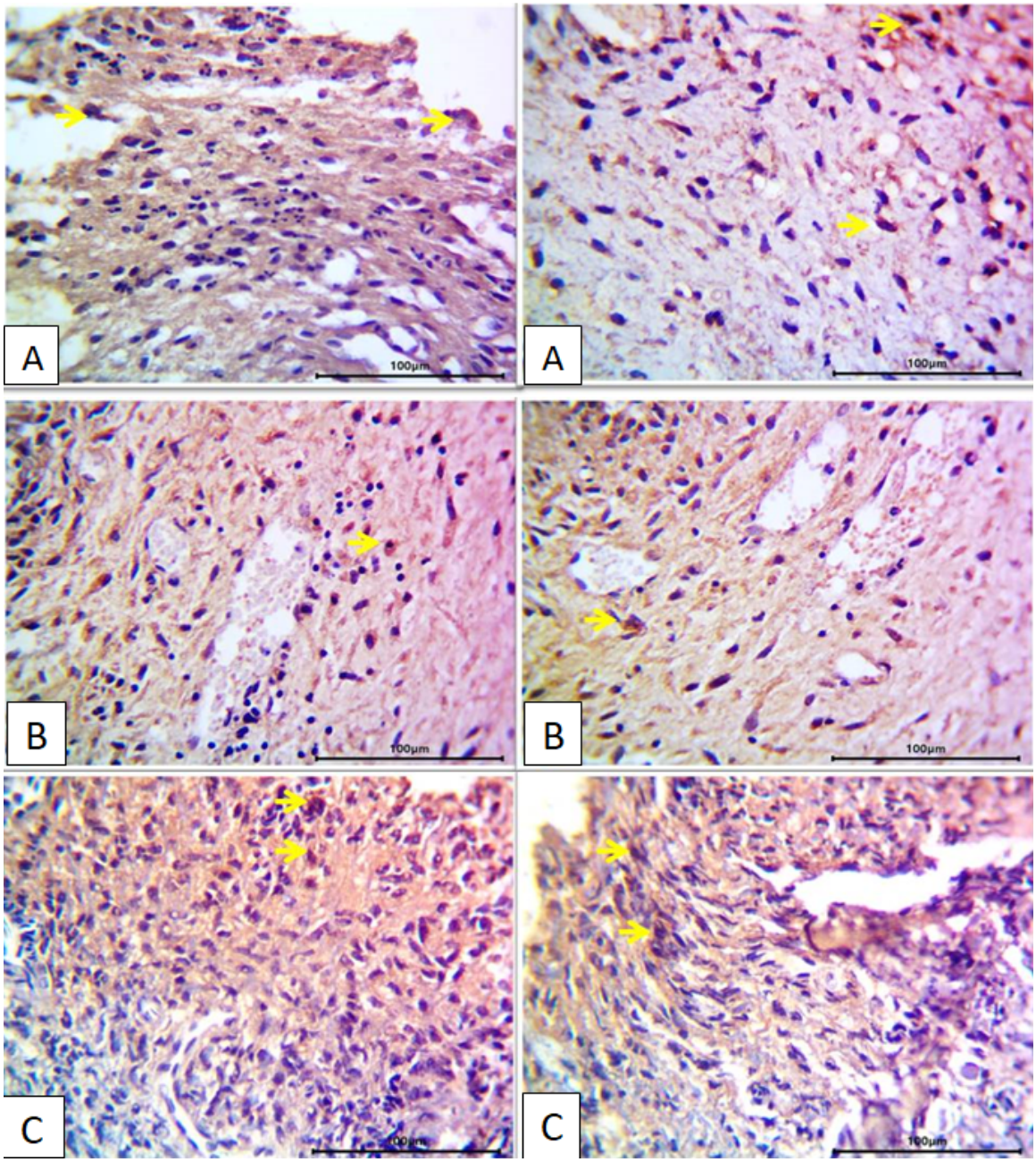


Figure 7. Representative composite photomicrographs of TGFβ1 immunoexpression in pulp tissue for the MTA + hyaluronic Acid (HA) group at days 1(A), 3(B), and 7(C) (×400 magnification). Mild staining persisted at days 1 and 3, with moderate expression evident at day 7.

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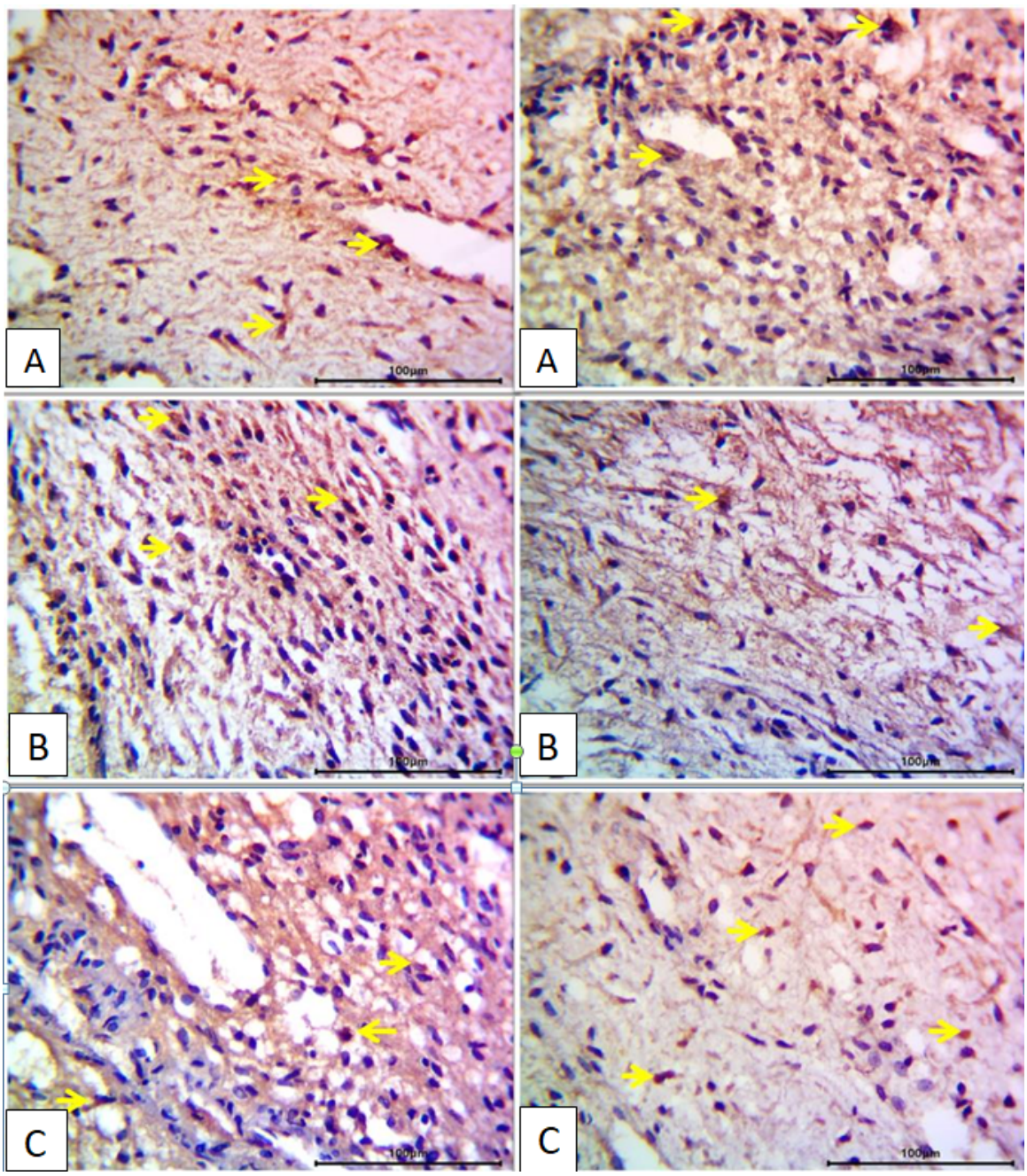


Figure 8. Representative composite photomicrographs of TGFβ1 immunoexpression in pulp tissue for the MTA + Transforming Growth Factor-β1 (TGFβ1) group at days 1(A), 3(B), and 7(C) (×400 magnification). Expression was strong at day 1, decreased to moderate at day 3, and increased again to strong at day 7.

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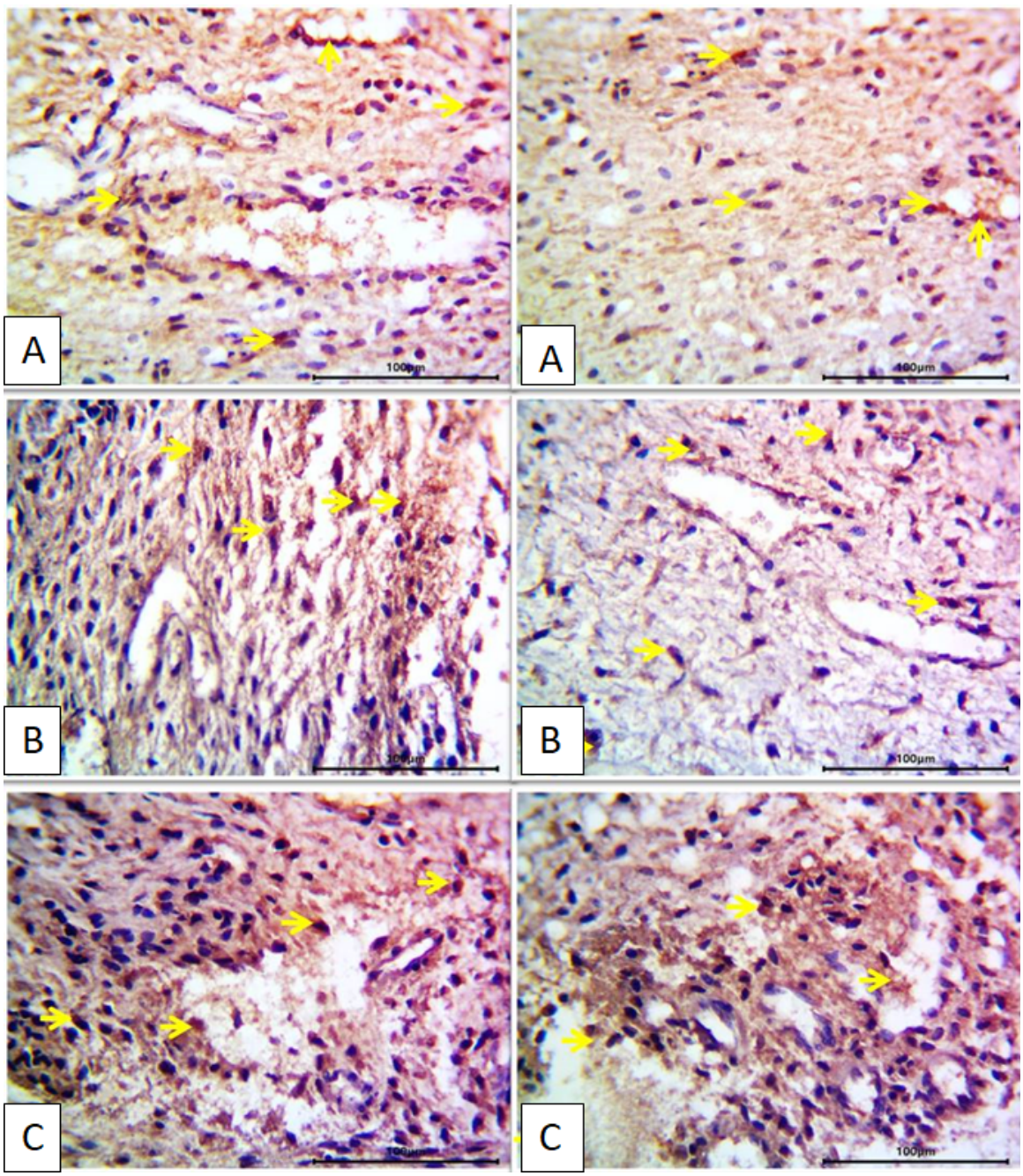


Figure 9. Representative composite photomicrographs of TGF-β1 immunoexpression in pulp tissue for the MTA + HA + TGFβ1 combination group at days 1(A), 3(B), and 7(C) (×400 magnification). The immunostaining was very strong at each time point suggesting that there was a significant degree of regenerative activity.